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(54) Title: FIBER SHAFT MODIFICATIONS FOR EFFICIENT TARGETING

(57) Abstract: Provided are adenoviral vectors and the production of such vectors. In particular, fiber shaft modifications for efficient targeting of adenoviral vectors are provided. The fiber shaft modifications can be combined with other modifications, such as fiber knob and/or penton modifications, to produce fully ablated (detargeted) adenoviral vectors. A scale-up method for the propagation of detargeted adenoviral vectors is also provided.

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FIBER SHAFT MODIFICATIONS FOR EFFICIENT TARGETING

RELATED APPLICATIONS

Benefit of priority is claimed to U.S. provisional application Serial No. 60/350,388, filed 24 January 2002, entitled "FIBER SHAFT MODIFICATIONS FOR EFFICIENT TARGETING," to Stevenson, Susan C., Kaleko, Michael, Smith, Theodore and Nemerow, Glen R., and to U.S. provisional application Serial No. 60/391,967, filed 26 June 2002, entitled "FIBER SHAFT MODIFICATIONS FOR EFFICIENT TARGETING," to Stevenson, Susan C., Kaleko, Michael, Smith, Theodore and Nemerow, Glen R. This application is also related to International PCT application No. (attorney docket number 22908-1236), filed the same day herewith, entitled "FIBER SHAFT MODIFICATIONS FOR EFFICIENT TARGETING," to Stevenson, Susan C., Kaleko, Michael, Smith, Theodore and Nemerow, Glen R. Where permitted, the subject matter of each of these applications is incorporated by reference herein.

FIELD OF INVENTION

The present invention generally relates to the field of adenoviral vectors and the production of such vectors. In particular, detargeted adenoviral vectors are provided.

BACKGROUND

Most, if not all, adenoviral vector-mediated gene therapy strategies aim to transduce a specific tissue, such as a tumor or an organ. Systemic delivery will require ablation of the normal virus tropism as well as addition of new specificities. Multiple interactions between adenoviral particles and the host cell are required to promote efficient cell entry (Nemerow (2000) *Virology 274*:1-4). An adenovirus entry pathway is believed to involve two separate cell surface events. First, a high affinity interaction between the adenoviral fiber knob and coxsackie-adenovirus receptor (CAR) mediates the attachment of the adenovirus particle to the cell surface. A subsequent association of penton with the cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which act as co-receptors, potentiates virus internalization. There are a plurality of adenoviral fiber receptors, which interact with the group B (*e.g.*, Ad3) and group C (*e.g.*, Ad5) adenoviruses. Both of these groups of adenoviruses appear to require interaction with integrins for

need to develop adenoviruses which are fully detargeted *in vivo* for use as a base vector for producing redirected adenoviruses.

Therefore, among the objects herein, it is an object herein to provide fully detargeted adenoviral vectors, methods for preparation thereof, and uses thereof.

SUMMARY

Detargeted and fully detargeted adenoviral particles, adenovirus vectors from which such particles are produced, methods for preparation of the vectors and particles and uses of the vectors and particles are provided. Provided and described are capsid modifications, such as fiber shaft modifications, and the resuling proteins that, when expressed on adenoviral particles provide for detargeting of adenoviral vectors. The capsid modifications, such as the fiber shaft modifications, can be combined with other modifications, such as fiber knob and/or penton modifications, to produce fully ablated (detargeted) adenoviral particles. Thus, adenoviral vectors and adenovirual particles whose native tropisms are ablated through a modification or modifications of capsid proteins, particularly a fiber shaft region, are provided.

Thus, provided are capsid mutiations, including fiber shaft modifications, that ablate binding to particular receptors, thereby permitting efficient targeting of adenoviral vectors that contain capsids with such modifications. For example, adenoviral vectors in which the fiber shaft's interaction with HSP is ablated (reduced or substantially eliminated), particuarly *in vivo*, are provided. These fiber shaft modifications can be combined with other modifications, such as fiber knob and/or penton modifications, to produce fully ablated (detargeted) adenoviral vectors. Also provided are retargeted vectors and particles that include a ligand or ligands to provide for targeting of the detargeted vectors and particles to selected cells and/or tissues. Retargeting can be effected, for example, by manipulating the fiber protein to redirect the receptor specificity to a particular cell type.

Also provided are nucleic acids encoding the modified fiber proteins and also modified penton proteins. Also provided are nucleic acids encoding the modified fiber shaft protein that has ablated HSP binding and combinations

mutations of individual amino acids in the fiber shaft that interact with HSP or mutations of amino acids in the fiber shaft that modify the ability of the HSP binding motif to interact with HSP. Adenoviral fiber shaft modifications also include replacements of fiber shafts using fiber shafts of adenoviruses, such as, for example, Ad3, Ad35 and Ad41 short fiber shaft, that do not contain HSP binding sites.

Also provided are adenoviral fiber shaft modifications that alter, particularly ablate viral interaction with HSP, as described above, in combination with fiber knob modifications that ablate viral interaction with CAR. The fiber knob modifications include: (a) mutations of individual amino acids in the fiber loop that interact with CAR, such as, for example, AB or CD loop modifications; (b) mutations of individual amino acids in the fiber loop that modify the ability of the CAR binding motif to interact with CAR; and (c) replacements of fiber knobs using adenoviruses that do not interact with CAR, such as, for example, Ad3 fiber knob, Ad41 short fiber knob, or Ad35 fiber knob.

Also provided are adenoviral fiber shaft modifications as described above in combination with penton modifications that ablate viral interaction with a_v integrins. The penton modifications include: (a) mutations of individual amino acids that interact with α_v integrins; (b) mutations of individual amino acids that modify the ability of the α_v integrin binding motif to interact with the α_v integrins; and (c) replacement of penton proteins using penton proteins from adenoviruses that do not interact with the α_v integrins.

Also provided are adenoviral fiber shaft modifications as described above in combination with fiber knob modifications as described above and penton modifications as described above.

Also provided is a scale-up method for the propagation of detargeted adenoviral vectors. The method uses polycations and/or bifunctional reagents, which when added to tissue culture medium results in entry of adenoviral particles into the producer cells.

Ad5 or Ad2 fiber, to produce a complete fiber whose binding to HSP is reduced or eliminated.

All of the modified capsids proteins provided herein also can include one or more further modifications that reduce or eliminate interaction of the resulting fiber with one or more cell surface proteins, such as but not limited to, CAR and α_v integrin or other receptor to which a particular native fiber binds, in addition to HSP. These modifications include, but are not limited to, modification to fiber that reduces or eliminates CAR binding and modification to penton that reduces or eliminate α_v integrin binding. The mutations can be in the fiber knob, shaft, tail and shaft, and also in penton.

Any and all of the modified capsid proteins provided herein can further include a ligand that binds to a particular receptor thereby endowing a fiber (or other capsid protein) with binding specificity or the ability to interact with such receptor. The ligand can be inserted into any suitable site in a capsid protein, such as an insertion or replacement. For example, fibers with ligands inserted into the knob region are exemplified. Any such ligand can be employed and a variety are exemplified herein.

A variety of modified capsid proteins are exemplified herein. These include, but are not limited to, fibers containing: the sequence of amino acids set forth in any of SEQ ID Nos. 52, 54, 56, 58, 62, 66, 70 and 72; or a sequence of amino acids having 60%, 70%, 80%, 90%, 95% or greater sequence identity with a sequence of amino acids set forth in any of SEQ ID Nos. 52, 54, 56, 58, 62, 66, 70 and 72; or a sequence of amino acids encoded by a sequence of nucleotides that hybridizes under conditions of high stringency along at least 70% of its length to a sequence of nucleotides that encodes a sequence of amino acids set forth in any of SEQ ID Nos. 52, 54, 56, 58, 62, 66, 70 and 72.

Nucleic acids encoding the capsid proteins, including the fibers are also provided. The nucleic acids can be provided as vectors, particularly as adenovirus vectors. Many adenoviral vectors are known and can be modified as needed in accord with the description herein. Adenoviral vectors include, but are not limited to, early generation adenoviral vectors, such as E1-deleted

prokaryotic cells, but typically are eukaryotic cells, including mammalian cells, such as primate, including human, cells. The cells can be of a specific type, such as a tumor cell or a cell in a particular tissue. The vectors can be oncolytic vector to effect killing of tumor cells.

Since the modified capsid proteins herein have reduced or eliminated binding to HSP, viral particles containing such proteins exhibit ablated binding to HSP *in vitro* and *in vivo*. Thus provided is a method of reducing transduction of cells that express HSP, such as hepatocytes in the liver, by modifying a capsid protein, such as fiber to eliminate or reduce interaction with or binding to HSP. Such reduction reduces or eliminates transduction of cells that express HSP, including liver cells.

Also provided are scale-up methods for the propagation of detargeted adenoviral particle, such as those provided herein. The method includes the steps of infecting or transducing a cell capable of replicating, maturing and packaging an adenoviral vector with a detargeted adenoviral vector in the presence of a reagent that results in entry of the adenoviral particle into the cell, such as a polycation and/or a bifunctional protein or other such reagent; and culturing the infected cell under conditions suitable for growth, spread and propagation of the adenoviral vector. The resulting adenoviral particles can be recovered. Polycations include, but are not limited to, hexadimethrine bromide, polyethylenimine, protamine sulfate and poly-L-lysine. Bifunctional proteins, include, but are not limited to, an anti-fiber antibody ligand fusion, an anti-fiber-Fab-FGF conjugate, an anti-penton-antibody ligand fusion, an anti-hexon antibody ligand fusion and a polylysine-peptide fusion. The ligand is selected to bind to a particular receptor.

The viral particles that express a modified capsids provided herein can be produced by this method. The modification include, for example, one or more mutations selected from among mutations that reduce or eliminate interactions with one or more of α_v integrins, coxsackie-adenovirus receptors (CAR) and heparin sulfate proteoglycans (HSP). Such mutations include, for example, PD1, KO1, KO12 and S*.

Figures 14A-14B shows the influence of fiber shaft mutations on *in vivo* adenoviral-mediated liver gene expression (Fig. 14A) and hexon DNA content (Fig. 14B).

Figures 15A-15B are plasmid maps of pSQ1HSPRGD (Fig. 15A) and pSQ1HSPKO1RGD (Fig. 15B).

Figure 16 shows that insertion of a RGD targeting ligand can restore transduction of the vectors containing the HSP binding shaft S* mutation.

Figures 17A-17B are plasmid maps of pSQ1AD35Fiber (Fig. 17A) and pSQ1Ad35FcRGD (Fig. 17B).

Figures 18A-18B are maps of plasmids encoding 35F chimeric fibers. Fig. 18A is a plasmid map of pSQ135T5H, and Fig. 18B is a plasmid map of pSQ15T35H.

Figure 19 shows the results of an *in vitro* analysis of Ad5 vectors containing Ad35 fibers and derivatives thereof.

Figure 20 shows the results of an *in vivo* analysis of Ad5 vectors containing Ad35 fibers and derivatives thereof.

Figures 21A-21B are plasmid maps of pSQ1Ad41sF (Fig. 21A) and pSQ1Ad41sFRGD (Fig. 21B).

Figure 22 shows the results of an *in vivo* analysis of Ad5 vectors containing Ad41 short fiber.

Figure 23 shows the *in vitro* analysis of Ad5 based vectors containing the Ad41 short fiber which has been re-engineered to contain a cRGD ligand in the HI loop.

Figure 24 shows enhanced transduction of AE1-2a cells with the Av3nBgFKO1 detargeted adenoviral vector using hexadimethrine bromide (HB), protamine sulfate (PS) and poly-lysine-RGD (K14) or the anti-penton-TNF*a* bifunctional protein (*a*pen-TNF).

Figure 25 shows ablation of HSP interaction decreases adenoviral-mediated gene transfer to other organs

Figure 26 shows *in vivo* liver transduction with adenoviral vectors which encode for B-galactosidase and contain various mutations to the fiber and/or penton proteins. Results are plotted as percent transduction as compared to

As used herein, "virus," "viral particle," "vector particle," "viral vector particle," and "virion" are used interchangeably to refer to infectious viral particles that are formed when, such as when a vector containing all or a part of a viral genome, is transduced into an appropriate cell or cell line for the generation of such particles. The resulting viral particles have a variety of uses, including, but not limited to, transferring nucleic acids into cells either *in vitro* or *in vivo*. For purposes herein, the viruses are adenoviruses, including recombinant adenoviruses formed when an adenovirus vector, such as any provided herein, is encapsulated in an adenovirus capsid. Thus, a viral particle is a packaged viral genome. An adenovirus viral particle is the minimal structural or functional unit of a virus. A virus can refer to a single particle, a stock of particles or a viral genome. The adenovirus (Ad) particle is relatively complex and may be resolved into various substructures.

Included among adenoviruses and adenoviral particles are any and all viruses that can be categorized as an adenovirus, including any adenovirus that infects a human or an animal, including all groups, subgroups, and serotypes. Thus, as used herein, "adenovirus" and "adenovirus particle" refer to the virus itself and derivatives thereof and cover all serotypes and subtypes and naturally occurring and recombinant forms, except where indicated otherwise. Included are adenoviruses that infect human cells. Adenoviruses can be wildtype or can be modified in various ways known in the art or as disclosed herein. Such modifications include, but are not limited to, modifications to the adenovirus genome that is packaged in the particle in order to make an infectious virus. Exemplary modifications include deletions known in the art, such as deletions in one or more of the E1a, E1b, E2a, E2b, E3, or E4 coding regions. Other exemplary modifications include deletions of all of the coding regions of the adenoviral genome. Such adenoviruses are known as "gutless" adenoviruses. The terms also include replication-conditional adenoviruses, which are viruses that preferentially replicate in certain types of cells or tissues but to a lesser degree or not at all in other types. For example, among the adenoviral particles provided herein, are adenoviral particles that replicate in abnormally proliferating tissue, such as solid tumors and other neoplasms. These include the viruses

"mask" the molecule and/or increase half-life, or conjugated to a non-viral protein.

As used herein, oncolytic adenoviruses refer to adenoviruses that replicate selectively in tumor cells

As used herein, a variety of vectors with different requirements and purposes are described. For example, one vector is used to deliver particular nucleic acid molecules into a packaging cell line for stable integration into a chromosome. These types of vectors also are referred to as complementing plasmids. A further type of vector carries or delivers nucleic acid molecules in or into a cell line (*e.g.*, a packaging cell line) for the purpose of propagating viral vectors; hence, these vectors also can be referred to herein as delivery plasmids. A third "type" of vector is the vector that is in the form of a virus particle encapsulating a viral nucleic acid and that is comprised of the capsid modified as provided herein. Such vectors also can contain heterologous nucleic acid molecules encoding particular polypeptides, such as therapeutic polypeptides or regulatory proteins or regulatory sequences to specific cells or cell types in a subject in need of treatment.

As used herein, the term "motif" is used to refer to any set of amino acids forming part of a primary sequence of a protein, either contiguous or capable of being aligned to certain positions that are invariant or conserved, that is associated with a particular function. The motif can occur, not only by virture of the primary sequence, but also as a consequence of three-dimensional folding. For example, the motif GXGXXG is associated with nucleotide-binding sites. In this fiber is a trimer, hence the trimeric structure can contribute fornation of a motif. Alternatively, a motif can be considered as a domain of a protein, where domain is a region of a protein molecule delimited on the basis of function without knowledge of and relation to the molecular substructure, as, e.g., the part of a protein molecule that binds to a receptor. As shown herein, the motif KKTK constitutes a consensus sequence for fiber shaft interaction with HSP.

As used herein, the term "bind" or "binding" is used to refer to the binding between a ligand and its receptor, such as the binding of an Ad5 shaft motif with HSP (Heparin Sulfate Proteoglycans), with a K_d in the range of 10-2

As used herein, the term "substantially eliminated" refers to a transduction efficiency less than about 11% of the efficiency of the wild-type fiber containing virus on HeLa cells. The transduction efficiency on Hela cells can be measured (see, *e.g.*, Example 1 of U.S. Patent Application Serial No. 09/870,203 filed on 30 May 2001, and published as U.S. Published application No. 20020137213, and of International Patent Application No. PCT/EP01/06286 filed 1 June 2001). Briefly, HeLa cells are infected with the adenoviral vectors containing mutated fiber proteins to evaluate the effects of fiber amino acid mutations on CAR interaction and subsequent gene expression. Monolayers of HeLa cells in 12 well dishes are infected with, for example, 1000 particles per cell for 2 hours at 37° C. in a total volume of, for example, 0.35 ml of the DMEM containing 2% FBS. The infection medium is then aspirated from the monolayers and I ml of complete DMEM containing 10% FBS was added per well. The cells are incubated for an sufficient time, generally about 24 hours, to allow for β-galactosidase expression, which is measured by a chemiluminescence reporter assay and by histochemical staining with a chromogenic substrate. The relative levels of β-galactosidase activity are determined using as suitable system, such as the Galacto-Light chemiluminescence reporter assay system (Tropix, Bedford, Mass.) Cell monolayers are washed with PBS and processed according to the manufacturer's protocol. The cell homogenate is transferred to a microfuge tube and centrifuged to remove cellular debris. Total protein concentration is determined, such as by using the bicinchoninic acid(BCA) protein assay (Pierce, Inc., Rockford, Ill.) with bovine serum albumin as the assay standard. An aliquot of each sample is then incubated with the Tropix β-galactosidase substrate for 45 minutes in a 96 well plate. A luminometer is used determine the relative light units (RLU) emitted per sample and then normalized for the amount of total protein in each sample (RLU/ug total protein). For the histochemical staining procedure, the cell monolayers are fixed with 0.5% glutaraldehyde in PBS, and then were incubated with a mixture of 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) per ml, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2 mM $MgCl_2$ in 0.5 ml of PBS. The monolayers are washed with PBS and the blue cells are visualized by light

provide complementing functions for the genes deleted in an adenoviral genome (*e.g.*, the nucleic acids encoding modified fiber proteins) and are able to package the adenoviral genomes into the adenovirus particle. The production of such particles require that the genome be replicated and that those proteins necessary for assembling an infectious virus are produced. The particles also can require certain proteins necessary for the maturation of the viral particle. Such proteins can be provided by the vector or by the packaging cell.

As used herein, detargeted adenoviral particles have ablated (reduced or eliminated) interaction with receptors with which native particles. Fully detargeted particles have two or more specificities altered. It is understood that *in vivo* no particles are fully ablated such that they do not interact with any cells. Degareted and fully degarated have reduced, typically substantiall reduced, or eliminated interaction with native receptors. For purposes herein, detargeted particles have reduced (2-fold, 5-fold, 10-fold, 100-fold or more) binding or virtually no binding to HSP receptors; fully degareted vectors include further capsid modifications to eliminate interactions with other receptors, such as CAR and integrins or other receptors. The particles still bind to cells, but the types of cells and interactions are reduced.

As used herein, pseudotyping describes the production of adenoviral vectors having modified capsid protein or capsid proteins from a different serotype than the serotype of the vector itself. One example, is the production of an adenovirus 5 vector particle containing an Ad37 or Ad35 fiber protein. This can be accomplished by producing the adenoviral vector in packaging cell lines expressing different fiber proteins. As provided herein, detargeting of an adenovirus 5 particle or other serotype group C adenovirus or other adenovirus that binds to HSP to reduce or eliminate binding to HSPs can be effected by replacing all or a portion that includes the shaft or at least the HSP consensus binding sequence of the Ad5 fiber with an adenovirus fiber or portion thereof that does not bind to HSP. Adenoviruses having fiber shafts that do not interact with HSP include (a) adenoviruses of subgroup B, e.g., Ad3, Ad35, Ad7, Ad11, Ad16, Ad21, Ad34 which do not have interaction with HSP, (b) adenoviruses of

substitution as follows: R512S, A515G, E516G, and K517G. Other KO mutations can be identified empirically or are known to those of skill in the art.

As used herein, PD mutations refer to mutations in the penton gene that ablate binding by the encoded to α_v integrin by replacing the RGD tripeptide. The PD1 mutation exemplified herein results in a substitution of amino acids 337 through 344 of the Ad5 penton protein, HAIRGDTF (SEQ ID No. 9), with amino acids SRGYPYDVPDYAGTS (SEQ ID No. 10), thereby replacing the RGD tripeptide.

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise beneficially altered.

As used herein, a therapeutically effective product is a product that is encoded by heterologous DNA that, upon introduction of the DNA into a host, a product is expressed that effectively ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures said disease.

As used herein, a subject is an animal, such as a mammal, typically a human, including patients.

As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The DNA is introduced into the selected target cells in a manner such that the heterologous DNA is expressed and a therapeutic product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy may also be used to deliver nucleic acid encoding a gene product to replace a defective gene or supplement a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid may encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product may be modified

program package (Devereux, J., *et al., Nucleic Acids Research 12(I)*:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al., J Molec Biol 215*:403 (1990); Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo *et al.* (1988) *SIAM J Applied Math 48*:1073). For example, the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. Other commercially or publicly available programs include, DNAStar "MegAlign" program (Madison, WI) and the University of Wisconsin Genetics Computer Group (UWG) "Gap" program (Madison WI)). Percent homology or identity of proteins and/or nucleic acid molecules can be determined, for example, by comparing sequence information using a GAP computer program (*e.g.*, Needleman *et al.* (1970) *J. Mol. Biol.* 48:443, as revised by Smith and Waterman ((1981) *Adv. Appl. Math.* 2:482). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986) *Nucl. Acids Res.* 14:6745, as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide.

As used herein, the term "at least 90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared, no more than 10% (i.e., 10 out of 100) of amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more

used. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which can be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

By way of example and not way of limitation, procedures using conditions of moderate stringency include, for example, but are not limited to, procedures using such conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 hours at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 hours at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which can be used are well-known in the art. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.1% SDS.

By way of example and not way of limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 minutes before autoradiography. Other conditions of high stringency which can be used are well known in the art.

Academic Press, Inc., Harcourt Brace Jaovanovich, NY; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal (1984), *A Practical Guide To Molecular Cloning*; *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.); *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Hogan *et al.* (1986) *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

B. Capsid modifications

Provided herein are modifications of the viral capsid that ablate the interaction of an adenovirus with its natural receptors. In particular, fiber modifications that result in ablation of the interaction of an adenvirus with HSP are provided. These fiber modifications can be combined with other capsid protein modifications, such as other fiber modifications and/or penton and/or hexon modifications, to fully ablate viral interactions with natural receptors, when expressed on a viral particle. The modification should not disrupt trimer formation or transport of fiber into the nucleus.

1. Fiber genes and proteins

The fiber protein extends from the capsid and mediates viral binding to the cell surface by binding to specific cell receptors (Philipson *et al.* (1968) *J. Virol. 2*:1064-1075). The fiber is a trimeric protein that includes an N-terminal tail domain that interacts with the adenovirus penton base, a central shaft domain of varying length, and a C-terminal knob domain that contains the cell receptor binding site (Chroboczek *et al.* (1995) *Curr.Top.Microbiol.Immunol. 199*:163-200; Riurok *et al.* (1990) *J.Mol.Biol. 215*:589-596; Stevenson *et al.* (1995) *J. Virol. 69*:2850-2857). The sequences of the fiber gene from a variety of serotypes including adenovirus serotypes 2 (Ad2), Ad5, Ad3, Ad35, Ad12, Ad40, and Ad41 are known. There are at least 21 different fiber genes in Genbank.

other mutations that alter the structure of the fiber shaft such that the HSP binding of the modified fiber protein is ablated when compared to the HSP binding of the wild-type fiber protein.

In a first aspect of this embodiment, an adenoviral fiber protein is modified by mutating one or more of the amino acids that interact with HSP. For example, the HSP binding motif of the modified fiber protein is no longer able to interact with HSP on the cell surface, thus ablating the viral interaction with HSP. For example, the adenoviral fiber is from a subgroup C adenovirus. Binding to HSP can be eliminated or reduced by mutating the fiber shaft in order to modify the ability of the HSP binding motif, which is, for example, KKTK sequence (SEQ ID No. 1) located between amino acid residues 91 to 94 in the Ad 5 fiber, to interact with HSP. The fiber proteins are modified by chemical and biological techniques known to those skilled in the art, such as site directed mutagenesis of nucleic aicd encoding the fiber or other techniques as illustrated herein.

In another aspect of this embodiment, the ability of a fiber to interact with HSP is modified by replacing the wild-type fiber shaft with a fiber shaft, or portion thereof, of an adenovirus that does not interact with HSP to produce chimeric fiber proteins. The portion is sufficient to reduce or eliminate interaction with HSP. Examples of adenoviruses having fiber shafts that do not interact with HSP include (a) adenoviruses of subgroup B, such as, but are not limited to, Ad3, Ad35, Ad7, Ad11, Ad16, Ad21, Ad34, which do not have interaction with HSP, (b) adenoviruses of subgroup F, such as, but are not limited to, Ad40 and Ad41, specifically the short fiber, and (c) adenoviruses of subgroup D, such as but are not limited to, Ad46. In another embodiment, adenoviral fiber shaft modifications that ablate viral interaction with HSP in combination with adenoviral fiber knob modifications that ablate viral interactions with CAR are provided. Suitable adenoviral fiber modifications include the fiber knob modifications are known to those of skill in the art and are exemplified herein (see, also, US. Patent Application Serial No. 09/870,203, filed on 30 May 2001, and published as U.S. Published application No. 20020137213, in International Patent Application No. PCT/EP01/06286 filed on 1 June 2001).

prepared using chemical and biological techniques known to those skilled in the art and as illustrated herein. Generally the adenovirus is a subgroup B or subgroup C adenovirus.

Preparation of fibers modified to eliminate or reduce HSP interactions and fibers modified to alter interactions with other receptors and cell surface proteins, such as CAR and/or α_v integrin, is also described in the Examples below. The nucleic acid and/or amino acid sequences of exemplary modified fibers, whose construction are described below) are set forth as SEQ ID Nos. 45-72 as follows:

SEQ ID Nos. 45 and 46 set forth the encoding nucleotide sequence and amino acid sequence of the modified fiber designated 5FKO1, where 5F refers to Adenovirus 5 fiber, KO1 is an exemplary mutation of the CAR interaction site described herein;

SEQ ID Nos. 47 and 48 set forth the encoding nucleotide sequence and amino acid sequence of the modified ber designated 5FKO1RGD, which further includes an RGD ligand to demonstrate retargeting;

SEQ ID Nos. 49 and 50 set forth the encoding nucleotide sequence and amino acid sequence of the modified fiber designated 5FKO12, where 5F refers to Adenovirus 5 fiber, KO12 is another exemplary mutation of the CAR interaction site described herein;

SEQ ID Nos. 51 and 52 set forth the encoding nucleotide sequence and amino acid sequence of the modified fiber designated 5F S* nuc, where 5F refers to Adenovirus 5 fiber, S* is an exemplary mutation of the shaft that alters binding to HSP;

SEQ ID Nos. 53 and 54 set forth the encoding nucleotide sequence and amino acid sequence of the modified fiber designated 5F S*RGD nuc, which further includes an RGD ligand;

SEQ ID Nos. 55 and 56 set forth the encoding nucleotide sequence and amino acid sequence of the modified ber designated 5FKO1S*, which contain the KO1 and S* mutations;

adenoviral vectors that express the modified capsid proteins and produce particles with modified fibers, or by packaging adenoviral vectors, particularly those that do not encode one or more capsid proteins in appropriate packaging lines. Hence, as discussed in detail below, adenoviral vectors and viral particles with modified fibers that do no bind to HSP are provided.

C. Nucleic acids, Adenoviral vectors and cells containing the nucleic acids and cells containing the vectors

Also provided are polynucleotides that encode modified capsid proteins and that encode vectors for preparation of adenovirus that express modified capsid proteins provided herein. The sequences of the wild-type adenovirus proteins are well known in the art and are modified as described herein. Nucleic acid molecules, such as cDNA that encode an exemplary modified fiber knob for ablated CAR interaction (see, SEQ ID No. 2 for KO1 and SEQ ID No. 3 for KO12) and for a modified penton for ablated a_v integrins (SEQ ID No. 4) are provided. As discussed above, modified capsid proteins with altered tropism for CAR and α_v integrins are known and described in the patents, applications and literature cited herein and known to those of skill in the art (see, *e.g.*, U.S. Patent No. 5,731,190, U.S. application Serial No. 09/870,203, published as U.S. Published application No. 20020137213; and Bai *et al.* (1993) *J. Virology* *67*:5198-5208).

Also provided are vectors including the polynucleotides provided herein. Such vectors include partial or complete adenoviral genomes and plasmids. Such vectors are constructed by techniques known to those skilled in the art and as illustrated herein. Also provided are adenoviral vectors modified by replacing whole fiber protein, or portions thereof, with the fiber proteins, or appropriate portions thereof, of an adenovirus that does not interact with HSP. Adenoviruses that do not interact with HSP can be identified by using the methods described herein which detect binding or non-binding of fiber proteins and adenoviruses with HSP. Among the adenoviral vectors provided herein are those of subgroup C, which include Ad2 and Ad5, in which the nucleic acid encoding the fiber shaft or a portion including the HSP-binding portion is

standard techniques. An exemplary method for producing adenoviral particles provided herein is as follows. The nucleic acid encoding the mutated fiber protein is made using standard techniques in an adenoviral shuttle plasmid. This plasmid contains the right end of the virus, in particular from the end of the E3 region through the right ITR. This plasmid is co-transfected into competent cells of an *E. coli* strain, such as the well known *E. coli* strain BJ5183 (see, *e.g.*, Degryse (1996) *Gene 170*:45-50) along with a plasmid, which contains the remaining portion of the adenovirus genome, except for the E1 region and sometimes also the E2a region and also contains a corresponding region of homology. Homologous recombination between the two plasmids generates a full-length plasmid encoding the entire adenoviral vector genome.

This full-length adenoviral vector genome plasmid is then transfected into a complementing cell line. The transfection can be performed in the presence of a reagent that directs adenoviral particle entry into producer cells. Such reagents include, but are not limited to, polycations and bifunctional reagents, such as those described herein. A complementing cell is, for example, is a cell of the PER.C6 cell line, which contains the adenoviral E1 gene (PER.C6 is available, for example, from Crucell, The Netherlands; deposited under ECACC accession no. 96022940; see, also Fallaux *et al.* (1998) *Hum. Gene Ther. 9*:1909-1907; see, also, U.S. Patent No. 5,994,128) or an AE1-2a cell (see, Gorziglia *et al.* (1996) *J. Virology 70*:4173-4178; and and Von Seggern *et al.* (1998) *J. Gen. Virol. 79*:1461-1468)).

AE1-2a cells are derivatives of the A549 lung carcinoma line (ATCC # CCL 185) with chromosomal insertions of the plasmids pGRE5-2.E1 (also referred to as GRE5-E1-SV40-Hygro construct and listed in SEQ ID No. 41) and pMNeoE2a-3.1 (also referred to as MMTV-E2a-SV40-Neo construct and listed in SEQ ID No. 42), which provide complementation of the adenoviral E1 and E2a functions, respectively.

The 633 cell line (see, von Seggern *et al.* (2000) *J. Virology 74*:354-362), which stably expresses the adenovirus serotype 5 wild-type fiber protein, and was derived from the AE1-2a cell line, is another an example of complementing cells. When the cell line is 633 cells, the final passage of

types, they can be amplified in cell lines derived from said cell type without provision of Ad complementary genes.

2. **Adenoviral vectors and particles**

The adenovirus as used herein for production of the adenoviral vectors and particles can be of any serotype. Adenoviral stocks that can be employed as a source of adenovirus or adenoviral coat protein, such as fiber and/or penton base, can be amplified from the adenoviral serotypes 1 through 47, which are currently available from the American Type Culture Collection (ATCC, Rockville, Md.), or from any other serotype of adenovirus available from any other source. For instance, an adenovirus can be of subgroup A (e.g., serotypes 12, 18, 31), subgroup B (e.g., serotypes 3, 7, 11, 14, 16, 21, 34, 35), subgroup C (e.g., serotypes 1, 2, 5, 6), subgroup D (e.g., serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47), subgroup E (serotype 4), subgroup F (serotype 40, 41), or any other adenoviral serotype.

In certain embodiments, the adenovirus is a subgroup B or a subgroup C adenovirus. Subgroup C adenoviruses which are modified in as described herein, include, but are not limited to, Ad2 and Ad5. For Ad5, the mutation is made in the KKTK sequence (SEQ ID No. 1) located between amino acid residues 91 to 94. The fiber proteins can be modified by chemical and biological techniques known to those skilled in the art. These methods include, but are not limited to, site directed mutagenesis and techniques as illustrated herein.

The adenoviral particle generally includes a targeting ligand as described above. The presence of the targeting ligand permits the delivery of a gene to a desired cell type which is different from the cell type that wild-type adenovirus particles infect or the same as that a wild-type particle infects, but allowing the infection in a selective manner, *i.e.*, non-target cell types are not significantly infected.

The adenoviral vectors provided herein can be used to study cell transduction and gene expression *in vitro* or in various animal models. The latter case includes *ex vivo* techniques, in which cells are transduced *in vitro* and then administered to the animal. They also can be used to conduct gene therapy on humans or other animals. Such gene therapy can be *ex vivo* or *in vivo*. For *in*

the packaging of the adenoviral vector particle. Such gutless adenoviral vector particles are recovered by standard techniques. The helper vector genome can be delivered in the form of a plasmid or similar construct by standard transfection techniques, or it can be delivered through infection by a viral particle containing the genome. Such viral particle is commonly called a helper virus. Similarly, the gutless adenoviral vector genome can be delivered to the cell by transfection or viral infection.

The helper virus genome can be the modified adenovirus vector genome as disclosed herein. Such genome also can be prepared or designed so that it lacks the genes encoding the adenovirus E1A and E1B proteins. In addition, the genome can further lack the adenovirus genes encoding the adenovirus E3 proteins. Alternatively, the genes encoding such proteins can be present but mutated so that they do not encode functional E1A, E1B and E3 proteins. Furthermore, such vector genome can not encode other functional early proteins, such as E2A, E2B3, and E4 proteins. Alternatively, the genes encoding such other early proteins can be present but mutated so that they do not encode functional proteins.

In producing the gutless vectors, the helper virus genome is also packaged, thereby producing helper virus. In order the minimize the amount of helper virus produced and maximize the amount of gutless vector particles produced, the packaging sequence in the helper virus genome can be deleted or otherwise modified so that packaging of the helper virus genome is prevented or limited. Since the gutless vector genome will have an unmodified packaging sequence, it will be preferentially packaged.

One way to do this is to mutate the packaging sequence by deleting one or more of the nucleotides comprising the sequence or otherwise mutating the sequence to inactivate or hamper the packaging function. One exemplary approach is to engineer the helper genome so that recombinase target sites flank the packaging sequence and to provide a recombinase in the packaging cell. The action of recombinase on such sites results in the removal of the packaging sequence from the helper virus genome. The recombinase can be provided by a nucleotide sequence in the packaging cell that encodes the recombinase. Such

to p53 is unnecessary. Thus, deletion of E1b-55KD should restrict vector replication to p53-deficient tumor cells.

Another approach is to use tumor-selective promoters to control the expression of early viral genes required for replication (see, *e.g.*, International PCT application Nos. WO 96/17053 and WO 99/25860). Thus, in this approach the adenoviruses selectively replicate and lyse tumor cells if the gene that is essential for replication is under the control of a promoter or other transcriptional regulatory element that is tumor-selective.

For example oncolytic adenoviral vectors that contain a cancer selective regulatory region operatively linked to an adenoviral gene essential for adenoviral replication are known (see, *e.g.*, U.S. Patent No. 5,998,205). Adenoviral genes essential for replication include, but are not limited to, E1a, E1b, E2a, E2b and E4. For example, an exemplary oncolytic adenoviral vector has a cancer selective regulatory region operatively linked to the E1a gene. In other embodiments, the oncolytic adenoviral vector has a cancer selective regulatory region of the present invention operatively linked to the E1a gene and a second cancer selective regulatory region operatively linked to the E4 gene. The vectors also can include at least one therapeutic transgene, such as, but not limited to, a polynucleotide encoding a cytokine such as GM-CSF that can stimulate a systemic immune response against tumor cells.

Other exemplary oncolytic adenoviral vectors include those in which expression of an adenoviral gene, which is essential for replication, is controlled by E2F-responsive promoters, which are selectively transactivated in cancer cells. Thus, vectors that contains an adenoviral nucleic acid backbone that contains in sequential order: A left ITR, an adenoviral packaging signal, a termination signal sequence, an E2F responsive promoter which is operably linked to a first gene, such as E1a, essential for replication of the recombinant viral vector and a right ITR (see, published International PCT application No. WO02/06786, and U.S. Patent No. 5,998,205).

In other embodiments, the oncolytic adenoviral vector has a cancer selective regulatory region operatively linked to the E1a gene and a second cancer selective regulatory region operatively linked to the E4 gene. The vectors

and helper viruses for use with helper-dependent vectors. The packaging cell line has heterologous DNA stably integrated into the chromosomes of the cellular genome. The heterologous DNA sequence encodes one or more adenovirus regulatory and/or structural polypeptides that complement the genes deleted or mutated in the adenovirus vector genome to be replicated and packaged. Packaging cell lines express, for example, one or more adenovirus structural proteins, polypeptides, or fragments thereof, such as penton base, hexon, fiber, polypeptide IIIa, polypeptide V, polypeptide VI, polypeptide VII, polypeptide VIII, and biologically active fragments thereof. The expression can be constitutive or under the control of a regulatable promoter. These cell lines are particularly designed for expression of recombinant adenoviruses intended for delivery of therapeutic products. For use herein, such packaging cell lines can express the modified capsid proteins, such as the fiber proteins who binding to HSP is reduced or eliminated, and/or the modified penton and hexon proteins.

Particular packaging cell lines complement viral vectors having a deletion or mutation of a DNA sequence encoding an adenovirus structural protein, regulatory polypeptides E1A and E1B, and/or one or more of the following regulatory proteins or polypeptides: E2A, E2B, E3, E4, L4, or fragments thereof.

The packaging cell lines are produced by introducing each DNA molecule into the cells and then into the genome via a separate complementing plasmid or plurality of DNA molecules encoding the complementing proteins can be introduced via a single complementing plasmid. Of interest herein, is a variation in which the complementing plasmid includes DNA encoding adenovirus fiber protein (or a chimeric or modified variant thereof), from Ad virus of subgroup D, such as Ad 37, polypeptide or fragment thereof.

For applications, such as therapeutic applications, the delivery plasmid further can include a nucleotide sequence encoding a heterologous polypeptide. Exemplary delivery plasmids include, but are not limited to, pDV44, pΔE1Bβ-gal and pΔE1sp1B. In a similar or analogous manner, therapeutic nucleic acids, such as nucleic acids that encode therapeutic genes, can be introduced.

Application No. PCT/EP01/06286. For different serotypes and strains of adenoviruses, loci for insertion of targeting ligands can be empirically determined. For different serotypes and strains, such loci can vary.

Because the adenovirus fiber has a trimeric structure, the ligand can be selected or designed to have a trimeric structure so that up to three molecules of the ligand are present for each mature fiber. Such ligands can be incorporated into the fiber protein using methods known in the art (see, *e.g.*, U.S. Patent No. 5,756,086). Instead of the fiber, the targeting ligand can be included in the penton or hexon proteins. Inclusion of targeting ligands in penton (see for example, in U.S. Patent Nos. 5,731,190 and 5,965,431) and in hexon (see for example, in U.S. Patent No. 5,965,541) is known.

In one exemplary embodiment, the ligand is included in a fiber protein, which is a fiber protein mutated as described herein. As shown herein, the targeting ligand can be included, for example, within the HI loop of the fiber protein. Any ligand that can fit in the HI loop and still provide a functional virus is contemplated herein. Such ligands can be as long as or longer than 80-100 amino acids (see, *e.g.*, Belousova *et al.* (2002) *J. Virol. 76*:8621-8631). Such ligands are added by techniques known in the art (see, *e.g.*, published International Patent Application publication No. WO99/39734 and U.S. Patent Application number 09/482,682). Other ligands can be be discovered through techniques known to those skilled in the art. Some non-limiting examples of these techniques include phage display libraries or by screening other types of libraries.

Targeting ligands include any chemical moiety that preferentially directs an adenoviral particle to a desired cell type and/or tissue. The categories of such ligands include, but are not limited to, peptides, polypeptides, single chain antibodies, and multimeric proteins. Specific ligands include the TNF superfamily of ligands which include tumor necrosis factors (or TNF's) such as, for example, TNFα and TNFβ, lymphotoxins (LT), such as LT-α and LT-β, Fas ligand which binds to Fas antigen; CD40 ligand, which binds to the CD40 receptor of B-lymphocytes; CD30 ligand, which binds to the CD30 receptor of neoplastic cells of Hodgkin's lymphoma; CD27 ligand, NGF ligand, and OX-40 ligand;

in sufficient quantities. This genetic engineering is accomplished by infecting the desired cell with an adenoviral particle whose genome includes a desired heterologous polynucleotide. The heterologous polynucleotide is then expressed in the genetically engineered cells. For use herein the cell is generally a mammalian cell, and is typically a primate cell, including a human cell. The cell can be inside the body of the animal (*in vivo*) or outside the body (*in vitro*). Heterologous polynucleotides (also referred to as heterologous nucleic acid sequences) are included in the adenoviral genome within the particle and are added to that genome by techniques known in the art. Any heterologous polynucleotide of interest can be added, such as those disclosed in U.S. Patent No. 5,998,205, incorporated herein by reference. Polynucleotides that are introduced into an Ad genome or vector can be any that encode a protein of interest or that are regulatory sequences. Proteins include, but are not limited to, therapeutic proteins, such as an immunostimulating protein, such as an interleukin, interferon, or colony stimulating factor, such as granulocyte macrophage colony stimulating factor (GM-CSF; see, *e.g.*, 5,908,763F. Generally, such GM-CSF is a primate GM-CSF, including human GM-CSF. Other immunostimulatory genes include, but are not limited to, genes that encode cytokines IL1, IL2, IL4, IL5, IFN, IFN, TNF, IL12, IL18, and flt3), proteins that stimulate interactions with immune cells (B7, CD28, MHC class I, MHC class II, TAPs), tumor-associated antigens (immunogenic sequences from MART-1, gp100(pmel-17), tyrosinase, tyrosinase-related protein 1, tyrosinase-related protein 2, melanocyte-stimulating hormone receptor, MAGE1, MAGE2, MAGE3, MAGE12, BAGE, GAGE, NY-ESO-1, -catenin, MUM-1, CDK-4, caspase 8, KIA 0205, HLA-A2R1701, -fetoprotein, telomerase catalytic protein, G-250, MUC-1, carcinoembryonic protein, p53, Her2/neu, triosephosphate isomerase, CDC-27, LDLR-FUT, telomerase reverse transcriptase, and PSMA), cDNAs of antibodies that block inhibitory signals (CTLA4 blockade), chemokines (MIP1, MIP3, CCR7 ligand, and calreticulin), and other proteins.

Other polynucleotides, including therapeutic nucleic acids, such as therapeutic genes, of interest include, but are not limited to, anti-angiogenic, and suicide genes. Anti-angiogenic genes include, but are not limited to, genes that

November 19, 1997, which published as PCT Publication No. WO/9925860), Nos, FasL, and sFasR (soluble Fas receptor).

Also contemplated are combinations of two or more transgenes with synergistic, complementary and/or nonoverlapping toxicities and methods of action. The resulting adenovirus can retain the viral oncolytic functions and, for example, additionally are endowed with the ability to induce immune and anti-angiogenic responses and other responses as desired.

Therapeutic polynucleotides and heterologous polynucleotides also include those that exert an effect at the level of RNA or protein. These include include a factor capable of initiating apoptosis, RNA, such as RNAi and other double-stranded RNA, antisense and ribozymes, which among other capabilities can be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, transcription factors, polymerases, genes encoding cytotoxic proteins, genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. trypsin, papain, proteinase K and carboxy-peptidase). Other polynucleotides include a cell or tissue specific promoters, such as those used in oncolytic adenoviruses (see, *e.g.*, U.S. Patent No. 5,998,205).

The heterologous polynucleotide encoding a polypeptide also can contain a promoter operably linked to the coding region. Generally the promoter is a regulated promoter and transcription factor expression system, such as the published tetracycline-regulated systems, or other regulatable systems (WO 01/30843), to allow regulated expression of the encoded polypeptide. Exemplary of other promoters, are tissue-selective promoters, such as those described in U.S. Patent No. 5,998,205. An exemplary regulatable promoter system is the Tet-On(and Tet-Off(systems currently available from Clontech (Palo Alto, CA). This promoter system allows the regulated expression of the transgene controlled by tetracycline or tetracycline derivatives, such as doxycycline. This system can be used to control the expression of the encoded polypeptide in the viral particles and nucleic acids provided herein. Other regulatable promoter systems are known (see, *e.g.*, published U.S. No. 20020168714, entitled "Regulation of Gene Expression Using Single-Chain,

U.S. Patent No. 5,998,205, U.S. Patent No. 5,801,029; U.S. patent application 60/348,670 and corresponding published International PCT application No. WO02/06786). These include the cytolytic, cytopathic viruses (or vectors), including the oncolytic viruses discussed above.

Alternatively, as discussed above, the vector can include a mutation or deletion in the E1b gene. Typically such mutation or deletion in the E1b gene is such that the E1b-19kD protein becomes non-functional. This modification of the E1b region can be combined with vectors where all or a part of the E3 region is present.

The oncolytic adenoviral vector can further include at least one heterologous coding sequence, such as one that encodes a therapeutic product. The heterologous coding sequence, such as therapeutic gene, is generally, although not necessarily, in the form of cDNA, and can be inserted at any locus that does not adversely affect the infectivity or replication of the vector. For example, it can be inserted in an E3 region in place of at least one of the polynucleotide sequences that encode an E3 protein, such as, for example, the 19kD or 14.7 kD E3 gene.

F. Propagation and Scale-up

Since doubly ablated adenoviral vectors containing mutations in the fiber and/or penton capsid proteins result in inefficient cell binding and entry via the CAR/αv integrin entry pathway, scaled up technologies improve the growth and propagation of such vectors to produce high titers of the adenoviral vectors for clinical use. Thus, also provided is a method for scaling up the production of detargeted adenoviral vectors. The detargeted adenoviral vectors comprise an adenoviral vector modified to ablate the interaction of said vector with at least one host cell receptor compared with a wild-type adenoviral vector. The detargeted adenoviral vectors can comprise an adenoviral vector modified to ablate the interaction of said vector with one, two, three or more host cell receptors. Thus, the method is suitable for producing the detargeted adenoviral vectors disclosed herein.

As noted, growth and propagation of doubly and fully ablated adenoviral vectors is enhanced by new scale up technologies. Doubly ablated vectors

Reagents which are useful in this method are those that are capable of directing adenoviral particle entry into the producer cells. Such reagents include, but are not limited to, polycations and bifunctional reagents. Suitable polycations include, but are not limited to, polytheylenimine; protamine sulfate; poly-L-lysine hydrobromide; poly(dimethyl diallyl ammonium) chloride (Merquat(r)-100, Merquat(r)280, Merquat(r)550); poly-L-arginine hydrochloride; poly-L-histidine; poly(4-vinylpyridine), poly(4-vinylpyridine) hydrochloride; poly(4-vinylpyridine)cross-linked, methylchloride quaternary salt; poly(4-vinyl-pyridine-co-styrene); poly(4-vinylpyridinium poly(hydrogen fluoride)); poly(4-vinyl-pyridinium-P-toluene sulfonate); poly(4-vinylpyridinium-tribromide); poly(4-vinyl-pyrrolidone-co-2-dimethylamino-ethyl methacrylate); polyvinylpyrrolidone, cross-linked; poly vinylpyrrolidone, poly(melamine-co-formaldehyde); partially methylated; hexadimethrine bromide; poly(Glu, Lys) 1:4 hydrobromide; poly(Lys, Ala) 3:1 hydrobromide; poly(Lys, Ala) 2:1 hydrobromide; poly-L-lysine succinylated; poly(Lys, Ala) 1:1 hydrobromide; and poly(Lys, Trp) 1:4 hydrobromide.

Suitable bifunctional reagents include, but are not limited to, antibodies or peptides that bind to the adenoviral capsid and that also contain a ligand that allows interaction with specific cell surface receptors of the producer cells. Examples of bifunctional reagents include: (a) anti-fiber antibody ligand fusions, (b) anti-fiber-Fab-FGF conjugate, (c) anti-penton-antibody ligand fusions, (d) anti-hexon antibody ligand fusions and (e) polylysine-peptide fusions. The ligand is any ligand that will bind to any cell surface receptor found on the producer cells.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

substitution of fiber amino acids 408 and 409, changing them from serine and proline to glutamic acid and alanine, respectively.

The vector was constructed as follows. First, the plasmid pSKO1 (Figure 1) was digested with the restriction enzymes SphI and MunI. The resulting DNA fragments were separated by electrophoresis on an agarose gel. The 1601 bp fragment containing all but the 5′ end of the fiber gene was excised from the agarose gel and the DNA was isolated and purified. The fragment was then ligated with the 9236 bp fragment of p5FloxHRFRGD, which had been digested with SphI and MunI. The resulting plasmid, p5FloxHRFKO1, was digested with SpeI and PacI and the 6867 bp fragment containing the fiber gene was isolated. The fragment was ligated with the 24,630 bp SpeI-PacI fragment of pNDSQ3.1. The resulting plasmid, pNDSQ3.1KO1 (Figure 2), was used together with pAdmireRSVnBg (Figure 3A) to generate a plasmid which encodes the full-length adenoviral vector genome. It, however, was necessary to remove the PacI site from pNDSQ3.1KO1 (Figure 2) prior to recombination with pAdmireRSVnBg (Figure 3A) so that the final plasmid contains a unique PacI site adjacent to the 5′ ITR. The PacI site in pNDSQ3.1KO1 was removed by digestion with PacI followed by blunting with T4 DNA Polymerase and religation. The resulting plasmid was called pNDSQ3.1KO1(Pac.

To generate a full-length plasmid containing the entire adenoviral genome, pAdmireRSVnBg (Figure 3A) was digested with SalI and co-transfected into competent cells of the *E. coli* strain BJ5183 along with pNDSQ3.1KO1ΔPac, which had been digested with BstBI. Homologous recombination between the two plasmids generated a full-length plasmid encoding the entire adenoviral vector genome, which was called pFLAv3nBgFKO1.

The plasmid pFLAv3nBgKO1 was linearized with PacI and transfected into 633 cells. In the fiber complementing 633 cell line, the resulting viral DNA containing the KO1 mutation is capable of being packaged into infectious viral particles containing a mixture of wildtype fiber and mutant fiber proteins. After five rounds of amplification in 633 cells, a cytopathic effect was observed. Three more rounds of amplification in 633 cells were performed followed by purification of the virus by standard CsCl centrifugation procedures. This viral

was digested with XbaI and EcoRI and cloned into pSQ1 using a three-way ligation to generate pSQ1KO12 (Figure 3C). The KO12 cDNA was incorporated into the genome of Av1nBg, an adenovirus vector with E1 and E3 deleted encoding β-galactosidase, by homologous recombination between ClaI-linearized pSQ1KO12 and pAdmireRSVnBg digested with SalI and PacI to generate Av1nBgKO12. The KO12 vector was transfected in 633 cells, scaled-up on non-fiber expressing cells and purified, as described above for KO1.

Av1nBgPD1

Genetic incorporation of the PD1 penton mutation to generate Av1nBgPD1

The adenoviral vector Av1nBgPD1 is an E1-, E3-deleted vector based on the adenovirus serotype 5 genome. It contains a RSV promoted nuclear-localizing β-galactosidase gene in the E1 region and also contains the PD1 mutation in the penton gene. The PD1 mutation results in a substitution of amino acids 337 through 344 of the penton protein, HAIRGDTF (SEQ ID No. 9), with amino acids SRGYPYDVPDYAGTS (SEQ ID No. 10), thus replacing the RGD tripeptide (see, Einfeld *et al.* (2001) *J. Virology 75*:11284-11291). The mutation in the penton gene was generated in the plasmid pGEMpen5, which contains the Adenovirus serotype 5 penton gene. To generate the mutation, four oligonucleotides were synthesized. The sequences of the oligonucleotides were as follows: penton 1: 5′ CGC GGA AGA GAA CTC CAA CGC GGC AGC CGC GGC AAT GCA GCC GGT GGA GGA CAT GAA 3′ (SEQ ID No. 11); penton 2: 5′ TAT CGT TCA TGT CCT CCA CCG GCT GCA TTG CCG CGG CTG CCG CGT TGG AGT TCT CTT CC 3′ (SEQ ID No. 12); penton 3: 5′ CGA TAG CCG CGG CTA CCC CTA CGA CGT GCC CGA CTA CGC GGG CAC CAG CGC CAC ACG GGC TGA GGA GAA GCG CGC 3′ (SEQ ID No. 13); penton 4: 5′ TCA GCG CGC TTC TCC TCA GCC CGT GTG GCG CTG GTG CCC GCG TAG TCG GGC ACG TCG TAG GGG TAG CCG CGG C 3′ (SEQ ID No. 14). The complementary oligonucleotides penton 1 and penton 2 were annealed to each other as were penton 3 and penton 4. The duplex generated by annealing penton 3 and penton 4 encoded the substitution of amino acids 337 through 344 described above. The duplex generated by annealing penton 1 and penton 2 possessed a 5 base

Av1nBgFKO1PD1

Genetic incorporation of the fiber KO1 or KO12 mutation in combination with the penton PD1 mutation to generate Av1nBgFKO1PD1

The adenoviral vectors Av1nBgFKO1PD1 and Av1nBgKO12PD1 were generated in an E1-, E3-deleted adenovirus serotype 5 genome. Both vectors contains a RSV promoted nuclear-localizing β-galactosidase gene in the E1 region and also contains either the KO1 or KO12 mutation in the fiber gene as well as the PD1 mutation in the penton gene. The vectors were constructed as follows. First, the plasmid pSQ1PD1 was digested with Csp45I and SpeI and the 23976 bp fragment containing the PD1 mutated penton gene was purified. In addition, the plasmids pSQ1KO1 or pSQ1KO12 (Figure 3B) were digested with Csp45I and SpeI and the 9090 bp fragment containing the KO1 or KO12 mutated fiber gene were purified. The appropriate purified fragments were ligated to each other to from the plasmid pSQ1FKO1PD1 (Figure 5A) or pSQ1KO12PD1 (Figure 5B) that contains the KO1 (or KO12) mutated fiber gene and the PD1 mutated penton gene. To generate virus, pSQ1FKO1PD1 or pSQKO12PD1 was linearized with ClaI and co-transfected into 633 cells with pAdmireRSVnBg (Figure 3A) which had been digested with SalI and PacI. After three rounds of amplification in 633 cells a cytopathic effect was observed and the crude viral lysate was then amplified on PerC6 cells. Hexadimethrine bromide was maintained in the medium at 4 μg/ml. Each virus was purified by standard CsCl centrifugation procedures.

EXAMPLE 2

***In Vitro* Evaluation of Adenoviral Vectors Containing the KO1 and PD1 Mutations**

Several recombinant adenoviral vectors were used in these studies to demonstrate the function of the KO1 fiber mutation and included Av1nBg, Av1nBgFKO1, Av1nBgPD1, and Av1nBgFKO1PD1, described above. The transduction efficiencies of adenoviral vectors containing the KO1 and/or PD1 mutations were evaluated on cells of the alveolar epithelial cell line A549. The transduction efficiencies were compared to that of Av1nBg, an adenoviral vector containing wild type fiber and penton.

kidney were collected from each animal. The median lobe of the liver was placed in neutral buffered formalin to preserve the sample for β-galactosidase immunohistochemistry. In addition, tissue from each organ was frozen to preserve it for hexon PCR analysis to determine vector content. A separate sample of liver from each mouse was frozen to preserve it for a chemiluminescent β-galactosidase activity assay.

For β-galactosidase immunohistochemistry slices of liver, approximately 2-3 mm thick, were placed in 10% neutral buffered formalin. After fixation, these samples were embedded in paraffin, sectioned, and analyzed by immunohistochemistry for β-galactosidase expression. A 1:1200 dilution was used of a rabbit anti-β-galactosidase antibody (ICN Pharmaceuticals, Inc.; Costa Mesa, CA) in conjunction with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) to visualize positive cells.

The chemiluminescent β-galactosidase activity assay was performed using the Galacto-Light PlusTM chemiluminescent assay (Tropix, Inc., Foster City, CA) system. Tissue samples were collected in lysis matrix tubes containing two ceramic spheres (Bio101, Carlsbad, CA) and frozen on dry ice. The tissues were thawed and 500 μl of lysis buffer from the Galacto-Light Plus kit was added to each tube. The tissue was homogenized for 30 seconds using a FastPrep System (Bio101, Carlsbad, CA). Liver samples were homogenized for an additional 30 seconds. β-galactosidase activity was determined in the liver homogenates according to the manufacture's protocol.

For hexon PCR analysis DNA from tissues was isolated using the Qiagen Blood and Cell Culture DNA Midi or Mini Kits (Qiagen Inc., Chatsworth, CA). Frozen tissues were partially thawed and minced using sterile disposable scalpels. Tissues were then lysed by incubation overnight at 55° C in Qiagen buffer G2 containing 0.2 mg/ml RNaseA and 0.1 mg/ml protease. Lysates were vortexed briefly and then applied to Qiagen-tip 100 or Qiagen-tip 25 columns. Columns were washed and DNAs were eluted as described in the manufacturer's instructions. After precipitation, DNAs were dissolved in water and the concentrations were spectrophotometrically determined (A260 and A280) on a

transduction compared to Av1nBg, suggesting that integrins are involved to some extent in hepatic uptake of the adenoviral vectors.

The results of the immunohistochemical staining of liver sections for β-galactosidase were consistent with the activity assays (data not shown) and demonstrate that gene expression was localized specifically to hepatocytes. The vectors containing the KO1 or KO12 mutation alone showed a slight increase in liver transduction as revealed by a more intense and frequent immunohistochemical-staining pattern. The vectors containing the PD1 mutation, either alone or combined with KO1 or KO12, showed little difference in transduction compared to Av1nBg. These results demonstrate that ablating the viral interaction with CAR and/or integrins is not sufficient to fully detarget adenoviral vectors from the liver *in vivo*.

In summary, the fiber AB loop mutation contained in Av1nBgFKO1 or Av1nBgKO12 ablates interaction with human and mouse CAR *in vitro* and diminished transduction *in vitro*. *In vivo*, however, fiber AB loop mutations behaved unexpectantly, because such mutations were found to enhance adenoviral-mediated gene transfer to liver and results in increasing vector potency. The penton base, PD1 mutation that ablates interaction with the second receptor involved in adenoviral internalization had no effect *in vitro* and little to no effect *in vivo*. These studies indicated that other receptors are responsible for adenoviral gene transfer to the liver *in vivo*.

EXAMPLE 4

Description Of Adenoviral Vectors Containing A Fiber With Amino Acid Substitutions At The Heparin Sulfate Binding Domain In The Fiber Shaft

Vectors containing substitutions at all four of the amino acids in the four amino acid motif in the Ad5 fiber shaft (residues 91 to 94, KKTK; SEQ ID No. 1) were generated in order to ablate the potential interaction with HSP. The mutation is termed HSP because it potentially eliminates binding to heparan sulfate proteoglycans. Vectors containing the HSP mutation alone and combined with the KO1 mutation (fiber knob AB loop mutation that ablates CAR binding), the PD1 mutation (penton mutation that eliminates RGD/integrin interaction), and a triple knockout vector (HSP, KO1, PD1) were generated.

To generate a recombinant adenoviral vector containing the HSP mutation in the fiber gene, pSQ1HSP was digested with ClaI and pAdmireRSVnBg (Figure 3A) was digested with SalI and PacI, then the two digested plasmids were co-transfected into 633 cells (von Seggern *et al.* (2000) *J Virology* 74:354-362). Homologous recombination between the two plasmids generated a full-length adenoviral genome capable of replication in 633 cells, which inducibly express Ad5 E1A and constitutively express wild-type fiber protein. After propagation on 633 cells, the virus capsid contained wildtype and mutant fiber proteins. To obtain viral particles containing only the modified fiber with the HSP mutation, the viral preparation was used to infect PerC6 cells, which do not express fiber. The resulting virus, termed Av1nBgFS*, was purified by standard CsCl centrifugation procedures.

Generation of vector containing the HSP and KO1 mutations

To generate an adenoviral vector containing the HSP and KO1 mutations in fiber, a PCR SOEing strategy identical to the one described above was used except that the plasmid pSQ1FKO1 was used as the template. The PCR SOEing product was digested with XbaI and MunI and ligated with the 6477 bp XbaI to MunI fragment of pFBshuttle(EcoRI) to generate pFBSEHSPKO1. The fiber gene containing the HSP and KO1 mutations was transferred from pFBSEHSPKO1 into the pSQ1 backbone using a three-way ligation strategy identical to the one described above for the HSP mutation alone, to generate the plasmid pSQ1HSPKO1 (Figure 10). Recombinant adenoviral vector containing the HSP and KO1 mutations in the fiber gene was generated by co-transfecting pSQ1HSPKO1 digested with ClaI and pAdmireRSVnBg digested with SalI and PacI into 633 cells. Adenovirus was propagated and purified as described above for the vector containing the HSP mutation alone. The resulting virus was termed Av1nBgFKO1S*.

5000, 10,000. HeLa cells were transduced with each of the above vectors, as well as a vector containing the KO1 mutation alone (Av1nBgFKO1) and a vector containing the PD1 mutation alone (Av1nBgPD1) at 2000 PPC. The cell monolayers were stained with X-gal 24 hours after infection and the percentage of cells expressing β-galactosidase was determined by microscopic observation and counting of cells. Transductions were done in triplicate and three random fields in each well were counted, for a total of nine fields per vector.

The results (depicted in Figures 13A-13B) showed significantly reduced transduction efficiencies on A549 and HeLa cells using vectors containing the HSP mutation compared to Av1nBg. The vectors containing the HSP mutations, however, demonstrated a dose response on A549 cells, in that increasing PPC ratios yielded increasing transduction.

Competition experiments were done to determine which receptor molecular interactions are involved in transduction of A549 cells by the various vectors. Transductions were performed in the presence or absence of various competitors including Ad5 fiber knob, a 50 amino acid oligopeptide derived from Adenovirus serotype 2 penton base which spans the RGD tripeptide region, or heparin (Invitrogen Life Technologies, Gaithersburg, MD). Monolayers of A549 cells were cultured in Richters medium supplemented with 10% FBS and were transduced with Av1nBg, Av1nBgS*, Av1nBgFKO1S*, Av1nBgS*PD1, or Av1nBgFKO1S*PD1 in infection medium (IM, Richters medium plus 2% FBS). Different PPC ratios were used for the different vectors to achieve measurable transduction levels. The PPC ratios were as follows: Av1nBg: 500 PPC, Av1nBgS*: 10,000 PPC, Av1nBgFKO1S*: 20,000 PPC, Av1nBgS*PD1: 10,000 PPC, and Av1nBgFKO1S*PD1: 20,000 PPC. Fiber knob competition was performed by pre-incubating cells in IM containing 16 μg/ml of fiber knob for 10 minutes at room temperature prior to infection with virus. Penton base peptide competition was performed by pre-incubating cells in IM containing 500nM peptide for 10 minutes at room temperature prior to infection with virus. Heparin competition was performed by pre-incubating each adenoviral vector in IM containing 3 mg/ml of heparin for 20 minutes at room temperature. In all cases, the competitor remained in the IM during the 1 hour infection when virus

separate sample of liver from each mouse was frozen to preserve it for a chemiluminescent β-galactosidase activity assay. β-galactosidase immunohistochemistry, hexon real-time PCR and the chemiluminescent β-galactosidase activity assay were carried out as described in Example 3. The results of the β-galactosidase activity assay (Figure 14A) and adenoviral hexon DNA content (Figure 14B) showed a dramatic reduction in liver transduction by vectors containing the HSP mutation. The vectors containing the HSP mutation alone resulted in reducing adenoviral-mediated liver gene expression by approximately 20-fold.When combined with the KO1 mutation (HSP, KO1, PD1), yielded approximately a 1000-fold reduction in β-galactosidase activity in the liver compared to the control vector Av1nBg. The vector containing the KO1 mutation alone showed a slight increase, on average, in liver transduction compared to Av1nBg, which is consistent with several previous experiments. The vectors containing the PD1 mutation alone or combined with KO1 showed a slight decrease in liver transduction compared to Av1nBg, although the decrease was not statistically significant. Analysis of hepatic adenoviral hexon DNA content (Figure 14B) confirmed these results.

The results of the immunohistochemical staining of liver sections for β-galactosidase were consistent with the activity assays (data not shown) and demonstrated that gene expression was localized specifically to hepatocytes. Vectors containing the HSP mutation, either alone or in combination with KO1 and/or PD1, showed a dramatic reduction in hepatocyte transduction. The vector containing the KO1 mutation alone showed a slight increase in liver transduction as revealed by a more intense and frequent immunohistochemical staining pattern. The vectors containing the PD1 mutation, either alone or combined with KO1, showed little difference in transduction compared to Av1nBg.

EXAMPLE 7

Description of Adenoviral Vectors Containing the HSP Fiber Shaft Mutation with and without the KO1 Fiber Mutation and with and without a cRGD Targeting Ligand in the Fiber Knob HI Loop

described in Example 1 above, was digested with BstXI and KpnI and the 4549 bp and 3156 bp fragments were isolated and purified. The three fragments were ligated to generate the plasmid pFBSEHSPKO1RGD, which encodes a fiber containing the HSP mutation, the KO1 mutation, and cRGD in the HI loop. The fiber gene from this plasmid was transferred into the pSQ1 backbone as follows. The plasmid pFBSEHSPKPO1RGD was digested with EcoRI and XbaI and the 7601 bp fragment was isolated and purified. The plasmid pSQ1 (Figure 3B) was digested with the restriction enzymes EcoRI, NdeI, and XbaI and the 16,431 bp EcoRI to NdeI fragment and the 9043 bp NdeI to XbaI fragment were isolated and purified. The three DNA fragments were ligated to generate the plasmid pSQ1HSPKO1RGD (Figure 15B).

To generate a recombinant adenoviral vector containing the HSP and KO1 mutations in the fiber gene along with a cRGD ligand in the HI loop, the plasmid pSQ1HSPKO1RGD was digested with ClaI and co-transfected into 633 cells with pAdmireRSVnBg which had been digested with SalI and PacI. After propagation on 633 cells, the virus capsid contained wildtype and mutant fiber proteins. To obtain viral particles containing only the modified fiber with the HSP and KO1 mutations and a cRGD ligand, the viral preparation was used to infect PerC6 cells, which do not express fiber. The resulting virus, termed Av1nBgFKO1S*RGD, was purified by standard CsCl centrifugation procedures.

EXAMPLE 8

In Vitro Evaluation of Adenoviral Vectors Containing the HSP Fiber Shaft Mutation with or without the Fiber Knob KO1 Mutation and with or without a cRGD Ligand in the HI Loop

The transduction efficiencies of adenoviral vectors containing the HSP fiber shaft mutation with or without the fiber KO1 mutation and with or without the cRGD ligand in the HI loop were evaluated on A549 cells. The transduction efficiencies were compared to that of Av1nBg, an adenoviral vector containing wild type fiber. The day prior to infection, cells were seeded into 24-well plates at a density of approximately 1 x 10^5 cells per well. Immediately prior to infection, the exact number of cells per well was determined by counting a representative well of cells. Each of the vectors, Av1nBg, Av1nBgS*,

corresponds to bp 25,308 through 25,334 of pSQ1. The DNA sequence of P-0006/L was as follows: 5′ TCT TGG TCA TCT GCA ACA ACA TGA AGA TAG TG 3′ (SEQ ID No. 18). It contains a 10 base pair 5′ extension that is complementary to the start of the Ad35 fiber gene, while the remainder of the primer anneals to the sequence immediately 5′ of the ATG start codon of the fiber gene in pSQ1. A PCR product of the expected size, 583 bp, was obtained and the DNA was gel purified. A second PCR amplified the Ad35 fiber gene using DNA extracted from wildtype Ad35 virus as a template. The primers used for this reaction were P-0007/U and 35FMun. The DNA sequence of P-0007/U was as follows: 5′ GT TGT TGC AG ATG ACC AAG AGA GTC CGG CTC A 3′ (SEQ ID No. 19). It contains a 10 base pair 5′ extension that is homologous to the 10 bp immediately prior to the ATG start codon of the fiber gene in Ad5. The remainder of the primer anneals to the start of the Ad35 fiber gene. The DNA sequence of 35FMun was as follows: 5′ AG CAA TTG AAA AAT AAA CAC GTT GAA ACA TAA CAC AAA CGA TTC TTT A GTT GTC GTC TTC TGT AAT GTA AGA A 3′ (SEQ ID No. 20). It contains a 46 base pair 5′ extension that is complementary to the region of the Ad5 genome between the end of fiber and the MunI site 40 bp downstream of the fiber gene. In addition, the 5′ extension encodes the last amino acid and stop codon of the Ad5 fiber gene. This region was retained in the vector because it contains the polyadenylation site for the fiber gene. The remainder of the primer anneals to the 3′ end of the Ad35 fiber gene, up to the next to last amino acid codon. A PCR product of the expected size, 1027 bp, was obtained and the DNA was gel purified. The two PCR products were mixed and joined together by PCR SOEing using primers P-0005/U and P-0009. The DNA sequence of P-0009 was as follows: 5′ AG CAA TTG AAA AAT AAA CAC GTT G 3′ (SEQ ID No. 21). It corresponds to bp 27,648 through 27,669 of pSQ1 and overlaps the MunI site in that region. A PCR product of the expected size, 1590 bp, was obtained and gel purified. It was cloned into the plasmid pCR4blunt-TOPO (Invitrogen Corporation, Carlsbad CA) using the Zero Blunt TOPO PCR Cloning Kit from Invitrogen. This intermediate cloning step simplified DNA sequencing of the PCR SOEing product. The resulting plasmid, termed pTOPOAd35F, was digested with XbaI and MunI

together. The DNA fragment generated in the second PCR was digested with Xba1 and Mun1 and was cloned directly into pFBshuttle(EcoR1) to create the fiber shuttle plasmid pFBshuttle5TS35H.

TABLE 2

Primers Used For The Exchange Of Fiber Shaft Regions Between Ad5 And Ad35 Fibers

Primer designation	Sequence	SEQ ID
P1	5'-GAACAGGAGGTGAGCTTAGA-3'	22
P2	5'-GTTAGGTGGAGGGTTTATTCCGGTCCAC AAAGTTAGCTTATC-3'	23
P3	5'-GATAAGCTAACTTTGTGGACCGGAATAAA CCCTCCACCTAAC-3'	24
P4	5'-GTGGCAGGTTGAATACTAGG-3	25
P5	5'-GTTAGGAGATGGAGCTGGTGTAGTCCATA AGGTGTTAATAC-3'	26
P6	5'-GTATTAACACCTTATGGACTACACCAGCT CCATCTCCTAAC-3'	27
P7	5'-TGCGCAAAAACAATCACCACGACAATCACAAT GTACATTGGAAGAAATCATACG-3'	28
P8	5'-ACATTGTGATTGTCGTGGTGATT GTTTTTGCGCATATGCCATACAATTTGAATG-3'	29

For the construction of the 35TS5H chimera, the pFBshuttleAd35 plasmid was used as the template with the P1 and P5 primers to generate the 5' fragment. The 3' fragment was generated using the pFBshuttle(EcoR1) plasmid as the template with the P6 and P4 primers. Following the same procedure described above, the fiber shuttle plasmid pFBshuttle35TS5H was generated.

For the 35TS5H and 5TS35H chimeras, the fiber gene was transferred from the pFBshuttle(EcoRI) backbone into pSQ1 as described above for the vector containing the Ad35 fiber. The resulting plasmids were called pSQ135T5H (Figure 18A) and pSQ15T35H (Figure 18B). In addition, adenoviral vectors were generated using the co-transfection strategy described above.

Construction of Ad5 vectors containing the Ad35 fiber with a cRGD targeting peptide in the HI loop of the 35F fiber knob: To incorporate the cRGD

Av1nBg35T5H vector behaves similarly to the Av1nBgS* vector *in vitro* and *in vivo*. These studies also demonstrate that vectors containing fiber proteins without an HSP binding site are fully viable.

EXAMPLE 11

5 *in Vivo* Evaluation Of Adenoviral Vectors Containing 35F And Derivatives Thereof

The objective of this study was to evaluate the *in vivo* biodistribution of adenoviral vectors containing 35F fibers and derivatives thereof to determine whether vectors containing these fibers ablate liver transduction due to their
10 shaft regions. A positive control cohort received Av1nBg and a negative control group received HBSS. Cohorts of five C57BL/6 mice received each vector via tail vein injection at a dose of 1×10^{13} particles per kg. The animals were sacrificed approximately 72 hours after vector administration by carbon dioxide asphyxiation. Liver, heart, lung, spleen, and kidney were collected from each
15 animal. The median lobe of the liver was placed in neutral buffered formalin to preserve the sample for β-galactosidase immunohistochemistry. In addition, tissue from each organ was frozen to preserve it for hexon PCR analysis to determine vector content. A separate sample of liver from each mouse was frozen to preserve it for a chemiluminescent β-galactosidase activity assay. β-
20 galactosidase immunohistochemistry, hexon real-time PCR and the chemiluminescent β-galactosidase activity assay were carried out as described in example 3.

The results of the β-galactosidase activity assay showed a dramatic reduction in liver transduction by vectors containing the Ad35 fiber or the
25 35T5H derivative (Figure 20) with an approximately 4- to 24-fold reduction in β-galactosidase activity in the liver compared to the control vector Av1nBg. These data demonstrate that shaft domains without HSP binding sites can effectively ablate hepatic *in vivo* gene transfer. In particular, HSP is the major entry mechanism for liver *in vivo*. CAR binding is a minor entry pathway.

5′ extension corresponding to the sequence in pSQ1 from the last codon of the fiber gene through the MunI site 40 bp downstream of the fiber gene. The remainder of the primer anneals to the 3′ end of the Ad41s fiber gene in pDV60Ad41sF. The PCR product was the expected size (1219 bp). The two PCR products were joined by PCR SOEing using primers P-0005/U and P-0009/L. The DNA sequence of P-0009/L was described above. The PCR SOEing reaction yielded the expected 1782 bp product. The product was cloned into pCR4blunt-TOPO to yield pCR4blunt-TOPOAd41sF. Next, pCR4blunt-TOPOAd41sF was digested with XbaI and MunI and the 1773 bp fragment containing the Ad41s fiber gene was gel purified. This fragment was ligated with the 6477 bp XbaI to MunI fragment of pFBshuttle(EcoRI) to generate pFBshuttleAd41sF. The Ad41s fiber gene was transferred into the pSQ1 backbone as follows. First, pFBshuttleAd41sF was linearized using EcoRI and this fragment was ligated with the 24,213 bp EcoRI fragment of pSQ1 to generate pSQ1Ad41sF (Figure 21A). Adenoviral vector containing the Ad41s fiber was generated using the co-transfection strategy described above.

Construction of Ad5 adenoviral vectors containing the Ad41 short fiber with a cRGD targeting ligand in the HI loop: A PCR SOEing strategy was used to generate a construct containing the Ad41s fiber with cRGD in the HI loop. The plasmid pFBshuttleAd41sF was used as a template for the PCR amplifications. First, a 1782 bp fragment was amplified using primers 5FF and 41sRGDR. The primer 5FF was described above. It anneals to pFBshuttleAd41sF at the XbaI site upstream of the fiber gene. The DNA sequence of the primer 41sRGDR was as follows: 5′ AGT ACA AAA ACA ATC ACC ACG ACA ATC ACA GTT TAT CTC GTT GTA GAC GAC ACT GA 3′ SEQ ID No. 34). It contains a 30 bp 5′ extension that encodes the cRGD targeting ligand. The remainder of the primer anneals to pFBshuttleAd41sF from bp 2878 through 2903. A second PCR amplified a 277bp region of pFBshuttleAd41sF using primers 3FR and 41sRGDF. The primer 3FR was described previously. It anneals to pFBshuttleAd41sF at the MunI site downstream of the fiber gene. The DNA sequence of 41sRGDF was as follows: 5′ TGT GAT TGT CGT GGT GAT TGT TTT TGT ACT AGT GGG TAT GCT TTT ACT TTT 3′ (SEQ ID No. 35). It contains a 30 bp 5′ extension that

chemiluminescent β-galactosidase activity assay was carried out as described in example 3.

The results of the hexon DNA analysis showed a dramatic reduction in liver transduction by vectors containing the Ad41sF fiber (Figure 22) with an approximately a 5-fold reduction in liver adenoviral DNA content compared to either control vector.

In the above examples, several novel adenoviral vectors were generated containing various fiber modifications designed to ablate the normal tropism of the vector. See Table 3. Vectors were generated in which the heparan sulfate binding domain in the fiber shaft was replaced by amino acid substitutions. This mutation, termed HSP, was also combined with the KO1 mutation (fiber knob AB loop mutation that ablates CAR binding), and the PD1 mutation (penton mutation that eliminates RGD/integrin interaction). In addition, a vector containing all three mutations (HSP, KO1, PD1) was generated. All vectors containing the HSP mutation, either alone or combined with other capsid modifications, showed dramatically reduced transduction efficiencies on A549 and HeLa cells. Furthermore, the same vectors showed dramatically reduced transduction of the liver following systemic delivery to mice. As an alternative strategy to ablate the normal tropism of Ad5-based vectors, the Ad5 fiber was replaced by a fiber from a different adenovirus serotype which does not bind CAR and does not contain the heparan binding domain in the shaft. Thus, vectors were generated containing the Ad35 fiber and the Ad41 short fiber. Versions of these two vectors containing a cRGD targeting ligand in the HI loop of the fiber were also produced. Additionally, vectors containing chimeric fibers were generated. A vector containing the Ad35 fiber tail and shaft regions fused to the Ad5 fiber knob domain as well as a vector containing the Ad5 fiber tail and shaft fused to the Ad35 fiber knob domain were constructed. Vectors containing either the entire Ad35 or Ad41 short fiber showed a significant reduction in liver transduction following delivery to mice via the tail vein. The observation of reduced liver transduction using vectors containing either an HSP mutation, the Ad35 fiber, or the Ad41 short fiber indicates the feasibility of detargeting adenoviral vectors *in vivo*. *In vitro* data with the Ad35 fiber or the Ad41 short

Vector	Description
Av1nBg41sFRGD	The same as Av1nBg but containing the full length Ad41 short fiber cDNA with a cRGD ligand in the HI loop of the Ad41 short fiber

EXAMPLE 14

In Vitro Evaluation Of Adenoviral Vectors Containing The Ad41sF With A cRGD Ligand In The HI Loop

The transduction efficiencies of adenoviral vectors containing the Ad41sF fiber with the cRGD ligand in the HI loop were evaluated on A549 cells. The transduction efficiencies were compared to that of Av1nBg, an adenoviral vector containing wild type fiber or Av1nBgFKO1RGD, an adenoviral vector containing the KO1 mutation in combination with the cRGD ligand in the HI loop. The day prior to infection, cells were seeded into 24-well plates at a density of approximately 1 x 10^5 cells per well. Immediately prior to infection, the exact number of cells per well was determined by counting a representative well of cells. Each of the vectors, Av1nBg, Av1nBgFKO1RGD, and Av1nBg41sFRGD were used to transduce A549 cells at a particle to cell ratios of 0 up to 10,000. The cell monolayers were stained with X-gal 24 hours after infection and the percentage of cells expressing β-galactosidase was determined by microscopic observation and counting of cells. Transductions were done in triplicate and three random fields in each well were counted, for a total of nine fields per vector. The results (Figure 23) show that the Av1nBg41sFRGD vector transduced cells to an equivalent level as Av1nBgFKO1RGD at all vector doses examined. Neither FKO1 or Ad41sF can bind CAR. The Ad41sF does not normally interact with CAR and additionally does not contain the HSP binding motif within the shaft domain. These data show that targeting peptides inserted into the loop regions of the fiber knob of KO1 and Ad41sF allows for transduction of target cells via the targeted receptor. Surprisingly, HSP, not CAR and integrins, is the major entry route *in vivo* and ablation of HSP binding permits targeting of adenoviral vectors.

significantly different than the Av1nBg (+) control using a unpaired, t-test analysis, P value (0.024. When expressed as a percent of the Av1nBg control values, the influence of each mutation, individually or in combination, becomes apparent. The S* mutation dramatically reduced gene transfer to all four organs, whereas, the KO1 mutation did not. Thus, the importance of the shaft for transduction *in vivo* extends to organs besides the liver. Finally, gene transfer to the lung, heart, and kidney was diminished with PD1 suggesting a role for integrin binding in vector entry in these organs.

EXAMPLE 16

Retargeting the S*, shaft modification and the 41sF fiber *in vivo*

Vectors containing the HSP mutation have been shown to effectively detarget adenoviral vectors *in vivo* (see examples 6 and 15). The objective of this study was to evaluate the ability to retarget vectors containing the S* modification or the Ad41sF to tumors *in vivo*. A cRGD peptide was genetically incorporated into the fiber HI loop and evaluated *in vitro* (Examples 8 and 14). These same vectors were then evaluated *in vivo* in tumor-bearing mice. Athymic nu/nu female mice were injected with 8 x 10^6 A549 cells on the right hind flank. When tumors reached approximately 100mm3 in size, they were randomized into treatment groups. Cohorts of 6 mice received each vector via tail vein injection at a dose of 1 x 10^{13} particles per kg. The animals were sacrificed approximately 72 hours after vector administration by carbon dioxide asphyxiation. Tumor, liver, heart, lung, spleen, and kidney were collected from each animal. Tissue from each organ was frozen to preserve it for real time PCR analysis to determine adenoviral hexon DNA content. Hexon real-time PCR was carried out as described in example 3. A separate sample of liver from each mouse was frozen to preserve it for a chemiluminescent β-galactosidase activity assay. Hexon real-time PCR and the chemiluminescent β-galactosidase activity assay was carried out as described in example 3.

The adenoviral vector biodistribution to the liver and tumor for each treatment group is shown in Figure 27. Vectors containing the S*, KO1S*, and 41sF fibers effectively detargeted the liver and tumor resulting in a significant reduction in the amount of adenoviral DNA found in each tissue in comparison to

prior to infection. The infection was carried out for 2 hrs. Complete medium containing hexadimethrine bromide at 4 μg/ml was added to each plate. Final concentration of hexadimethrine bromide in all of these experiments was maintained at 4 μg/ml. The titers were determined spectrophotometrically using the conversion of 1OD at A260nm per 1 x 10^{12} particles (Mittereder *et al.* (1996) *J Virology 70*:7498-7509). The total particle yield was then normalized for the number of plates used for transduction.

The inclusion of hexadimethrine bromide in the medium during the course of infection allows for the efficient propagation of detargeted adenoviral vectors containing fiber and penton mutations either alone or in combination. The affect of hexadimethrine bromide on vector yields is shown in Table 4. A 35-fold improvement in the yield of Av3nBgFKO1 was found when hexadimethrine bromide was included in the culture medium and resulted in increasing the vector yield from 1.3 x 10^{10} up to 4.6 x 10^{11} vector particle per plate. Hexadimethrine bromide has a minimal effect on the yield of the Av1nBgPD1 adenoviral vector containing the penton, PD1 mutation with only a 1.2 fold improvement. The greatest effect using hexadimethrine bromide was found on the propagation of the doubly ablated adenoviral vector, Av1nBgFKO1PD1 with increases in vector yield from barely detectable levels up to 4.53 x 10^{10} vector particles per plate. These data demonstrate that use of nonspecific entry mechanisms allows for the efficient scale-up of detargeted adenoviral vectors.

TABLE 4

Efficient Scale-Up Of Detargeted Adenoviral Vectors Using hexadimethrine bromide

Vector	Vector Yield (particles/plate)		Fold Improvement
	(-) hexadimethrine bromide	(+) hexadimethrine bromide	
Av1nBg	3.89 x 10^{11}	5.72 x 10^{11}	1.47
Av3nBg	8.58 x 10^{10}	2.38 x 10^{11}	2.77
Av3nBgFKO1	1.30 x 10^{10}	4.60 x 10^{11}	35.4
Av1nBgPD1	1.95 x 10^{11}	2.40 x 10^{11}	1.23
Av1nBgFKO1PD1	TLTC*	4.53 x 10^{10}	†

	10 ppc - anti-penton TNF	10 ppc + anti-penton TNF	100 ppc - anti-penton TNF	100 ppc + anti-penton TNF
	Percentage of CPE			
48 h	20-30%	20-30%	90-100%	90-100%
72 h	60-70%	80-90%	100%	100%
120 h	100%	100%	100%	100%
Av3nBgKO1 24hrs				
24 h	0%	0%	0%	0%
48 h	0%	10-20%	0%	90-100%
72 h	5%	60-70%	5%	100%
120 h	40-50%	100%	100%	100%

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

the portion comprises a sufficient portion to alter HSP binding of the resulting protein.

11. The modified protein of claim 10, wherein the binding to HSP of the modified fiber protein is eliminated or reduced compared to the unmodified protein.

12. The modified protein of claim 10, wherein the remainder of the fiber protein is from the second adenovirus.

13. The modified protein of any of claims 2, 3, 10 and 11, further comprising one or more further modifications that reduce or eliminate interaction of the resulting fiber with one or more cell surface proteins in addition to HSP.

14. The modified protein of claim 13, further comprising a ligand, whereby the resulting fiber binds to a receptor for the ligand.

15. The modified protein of claim 14, wherein the ligand is included in the knob region.

16. The modified protein of claim 14, wherein the ligand is inserted or it replaces a portion of the fiber, whereby the resulting fiber binds to a receptor for the ligand.

17. A modified protein of claim 11, wherein affinity for HSP is reduced at least by an amount selected from among reduced 5-fold, 10-fold and 100-fold.

18. The modified protein of claim 11, wherein the first adenovirus is selected from the group consisting of subgroup B, D or F, and the second is of subgroup C.

19. The modified protein of claim 10, wherein the first adenovirus is selected from the group consisting of Ad3, Ad35, Ad7, Ad11, Ad16, Ad21, Ad34, Ad40, Ad41 and Ad46.

20. The modified protein of claim 18, wherein the second adenovirus is Ad5 or Ad2.

21. The modified protein of claim 19, wherein the second adenovirus is Ad5 or Ad2.

22. A modified protein of claim 1 selected from the group consisting of a fiber protein comprising:

37. An adenoviral particle, comprising a modified protein of any of claims 1-12 and 14-22, whereby binding of the viral particle to HSP is altered compared to a particle that expresses an unmodified fiber.

38. An adenoviral particle, comprising a modified protein of claim 13, whereby binding of the viral particle to HSP is altered compared to a particle that expresses an unmodified fiber.

39. An adenoviral particle of claim 37, wherein a native receptor for the fiber is coxsackie-adenovirus receptor (CAR).

40. The adenoviral particle of claim 39, further comprising a mutation in the CAR-binding region of the capsid.

41. The adenoviral particle of claim 39, further comprising a mutation in the α_v integrin-binding region of the capsid, whereby binding to the integrin is eliminated or reduced.

42. The adenoviral particle of claim 40, further comprising a mutation in the α_v integrin-binding region of the capsid, whereby binding to the integrin is eliminated or reduced

43. The adenoviral particle of claim 39, wherein the CAR-binding region of the capsid modified is on a fiber knob.

44. The adenoviral particle of claim 43, wherein the fiber knob modification is in the AB loop or CD loop.

45. The adenoviral particle of claim 44, wherein the fiber knob modification is selected from the group consisting of KO1 and KO12.

46. The adenoviral particle of claim 39, wherein the adenovirus is a subgroup C, D or F adenovirus.

47. The adenoviral particle of claim 46, wherein the subgroup C virus is Ad2 or Ad5, the subgroup D virus is Ad46 and the subgroup F virus is Ad41.

48. The adenoviral vector of claim 27 that is an early generation adenoviral vector, a gutless adenoviral vector or a replication-conditional adenoviral vector.

49. The adenoviral vector of claim 28 that is an early generation adenoviral vector, a gutless adenoviral vector or a replication-conditional adenoviral vector.

the adenoviral vector is an oncolytic vector; and

the cell is killed .

66. The method of claim 64, wherein the cell is a mammalian cell.

67. The method of claim 64, wherein the cell is a primate cell.

68. The method of claim 67, wherein the cell is a human cell.

69. A method of reducing transduction of liver cells by an adenoviral particle, comprising reducing or eliminating binding of the particle to heparin sulfate proteoglycans (HSPs) on the liver cells.

70. A scale up method for the propagation of a detargeted adenoviral particle, comprising:

infecting a cell capable of replicating, maturing and packaging an adenoviral vector with a detargeted adenoviral vector in the presence of a reagent that results in entry of the adenoviral particle into the cell;

culturing the infected cell under conditions suitable for growth, spread and propagation of the adenoviral vector; and

recovering the resulting adenoviral particles.

71. The method of claim 70, wherein the reagent is a polycation.

72. The method of claim 71, wherein the polycation is selected from the group consisting of hexadimethrine bromide, polyethylenimine, protamine sulfate and poly-L-lysine.

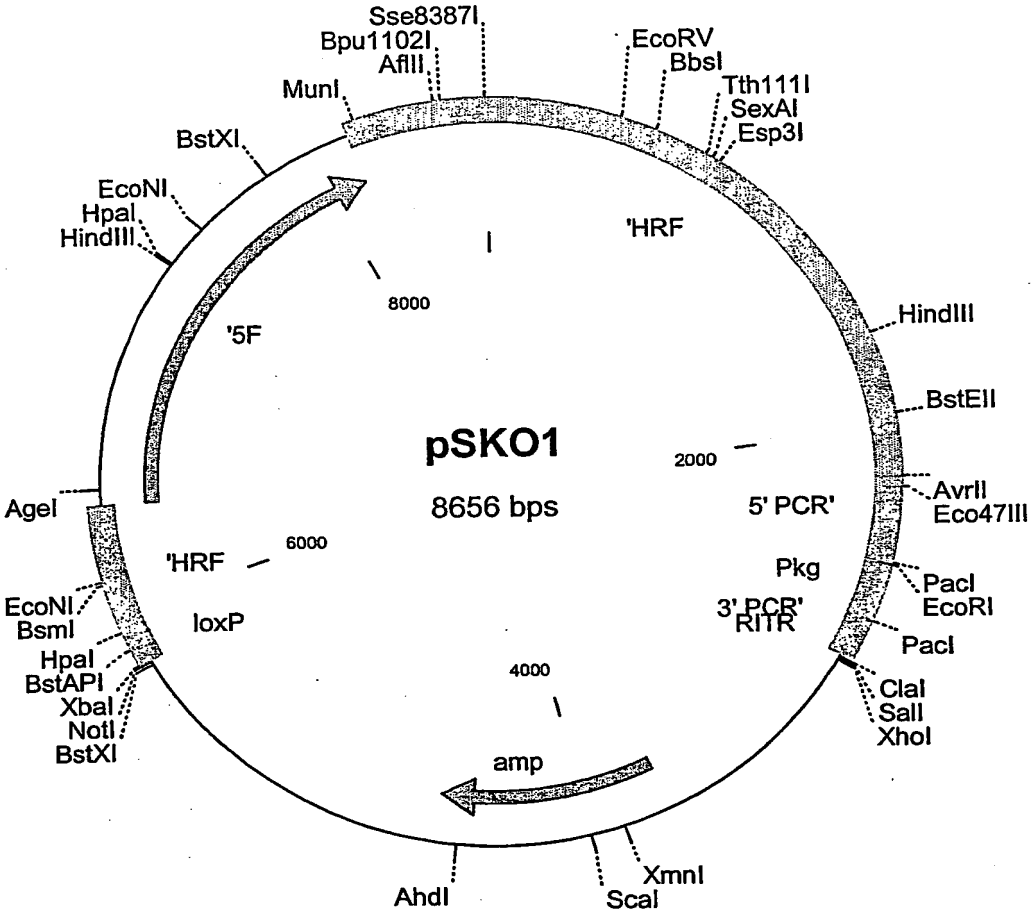
73. The method of claim 70, wherein the reagent is a bifunctional protein that binds to the adenoviral particle and to a receptor on the cell.

74. The method of claim 73, wherein:

the bifunctional protein is selected from the group consisting of an anti-fiber antibody ligand fusion, an anti-fiber-Fab-FGF conjugate, an anti-penton-antibody ligand fusion, an anti-hexon antibody ligand fusion and a polylysine-peptide fusion, wherein the ligand is a ligand that binds to the receptor.

75. The method of any one of claims 70-74, wherein the detargeted adenoviral particle expresses a modified capsid, whereby binding to at least one host cell receptor is reduced or eliminated compared with a wild-type adenovirus.

Figure 1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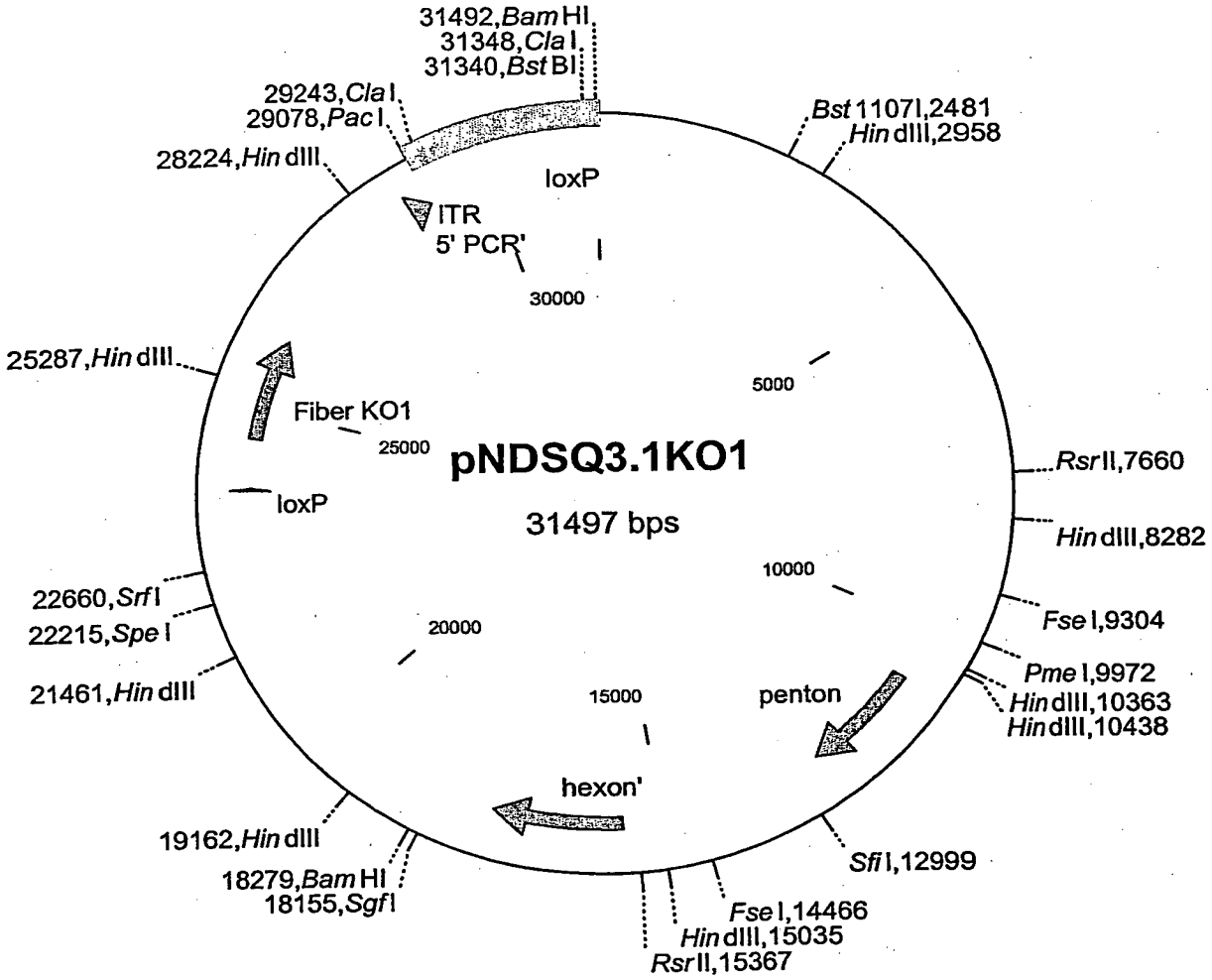
8656 bps
Sse8387I
Bpu1102I
AflII
EcoRV
BbsI
Tth111I
SexAI
Esp3I
MunI
BstXI
EcoNI
HpaI
HindIII
'HRF
'5F
8000
HindIII
BstEII
2000
AvrII
Eco47III
5' PCR'
AgeI
6000
'HRF
Pkg
PacI
EcoRI
EcoNI
BsmI
loxP
3' PCR'
RITR
PacI
HpaI
BstAPI
XbaI
NotI
BstXI
4000
ClaI
SalI
XhoI
amp
AhdI
ScaI
XmnI

Figure 2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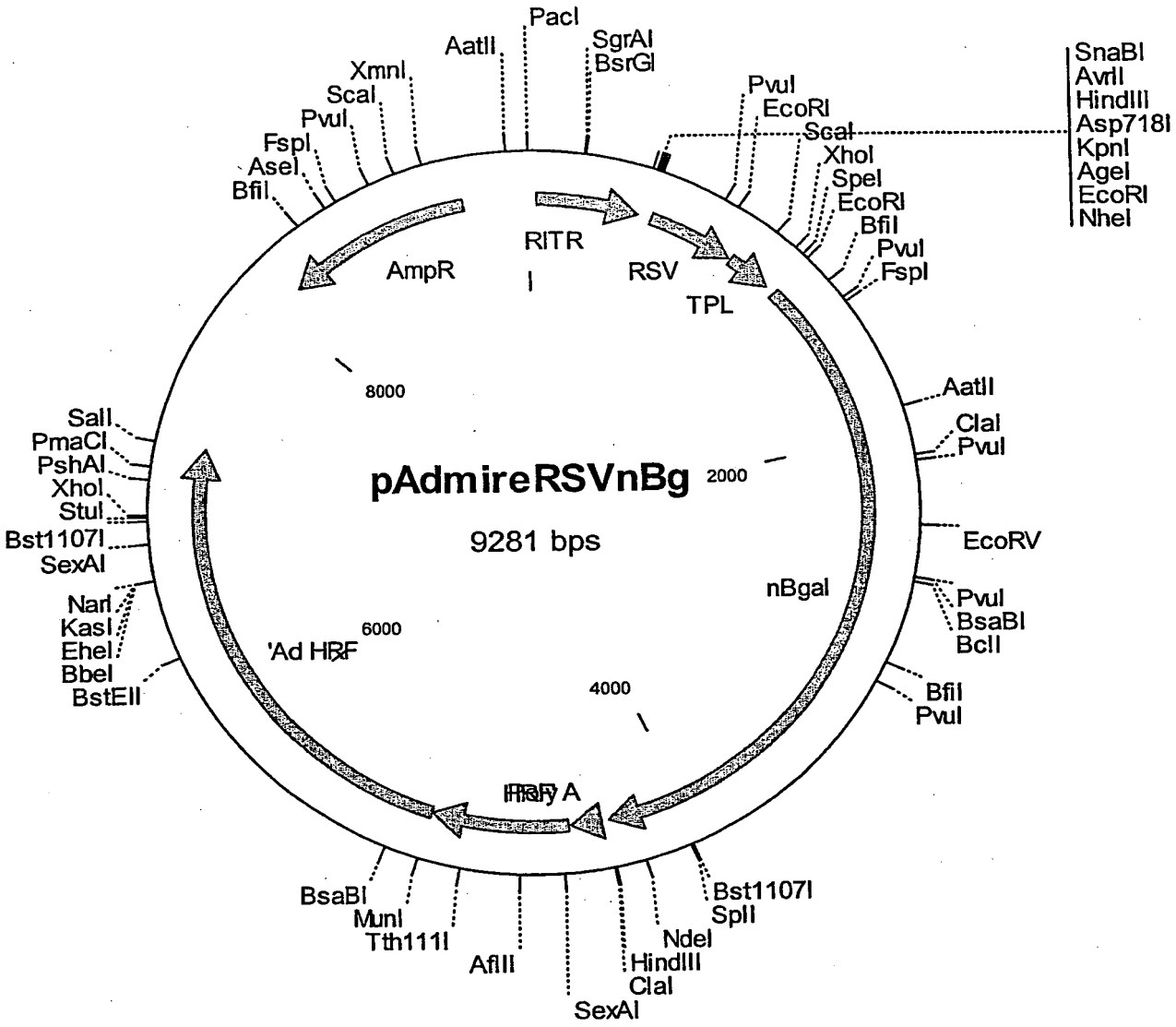
31348,ClaI
31340,BstBI
29243,ClaI
29078,PacI
28224,HindIII
loxP
ITR
5' PCR'
30000
25287,HindIII
Fiber KO1
25000
pNDSQ3.1KO1
31497 bps
loxP
22660,SrfI
22215,SpeI
21461,HindIII
20000
19162,HindIII
18279,BamHI
18155,SgfI
15000
hexon'
5000
BstI1107I,2481
HindIII,2958
RsrII,7660
HindIII,8282
10000
FseI,9304
PmeI,9972
HindIII,10363
HindIII,10438
penton
SfiI,12999
FseI,14466
HindIII,15035
RsrII,15367

Figure 3A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2000
4000
6000
8000
AmpR
RITR
RSV
TPL
nBgal
'Ad HRF
HRF A
PacI
AatII
SgrAI
BsrGI
XmnI
ScaI
PvuI
FspI
AseI
BfiI
PvuI
EcoRI
ScaI
XhoI
SpeI
EcoRI
BfiI
PvuI
FspI
SnaBI
AvrII
HindIII
Asp718I
KpnI
AgeI
EcoRI
NheI
AatII
ClaI
PvuI
EcoRV
PvuI
BsaBI
BclI
BfiI
PvuI
Bst1107I
SplI
NdeI
HindIII
ClaI
SexAI
AflII
BsaBI
MunI
Tth111I
SalI
PmaCI
PshAI
XhoI
StuI
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SexAI
NarI
KasI
EheI
BbeI
BstEII

Figure 3B

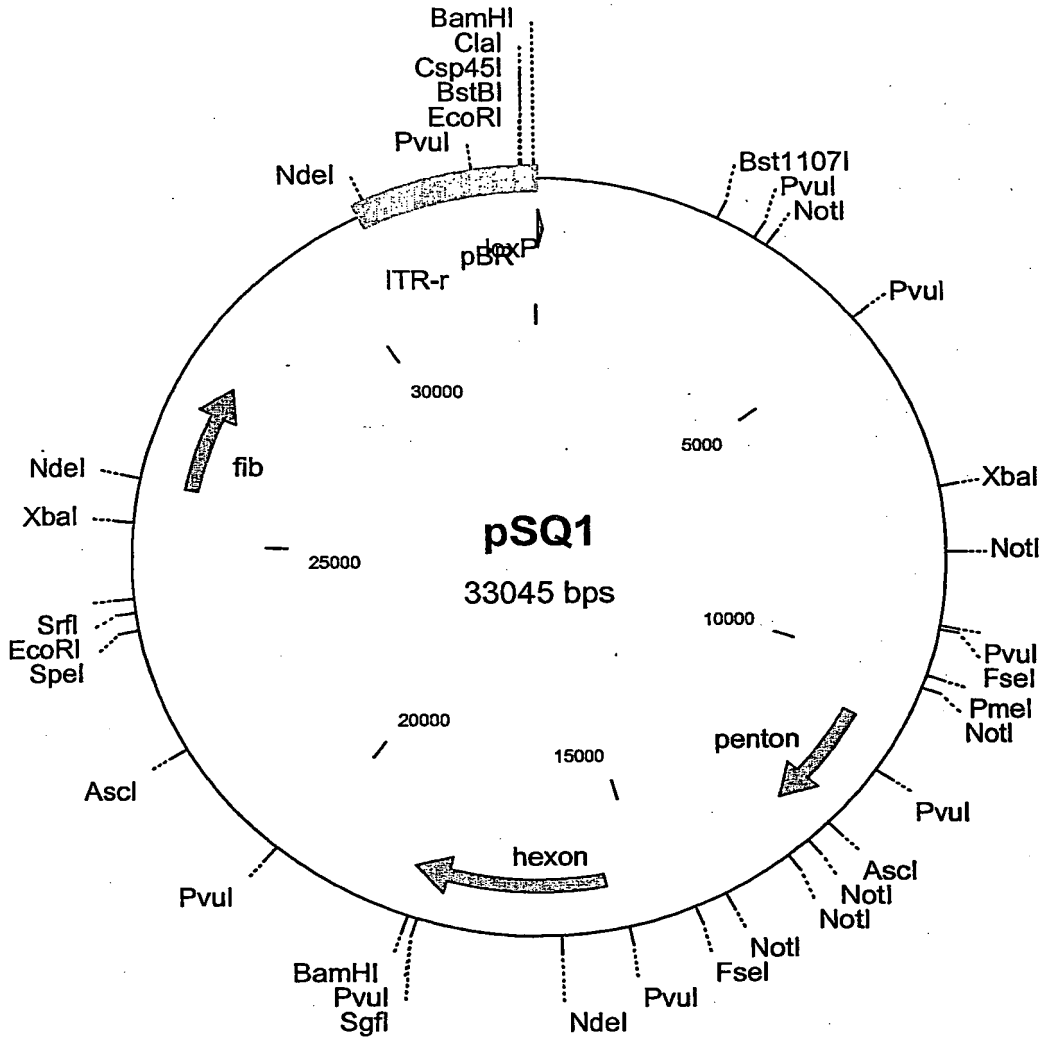
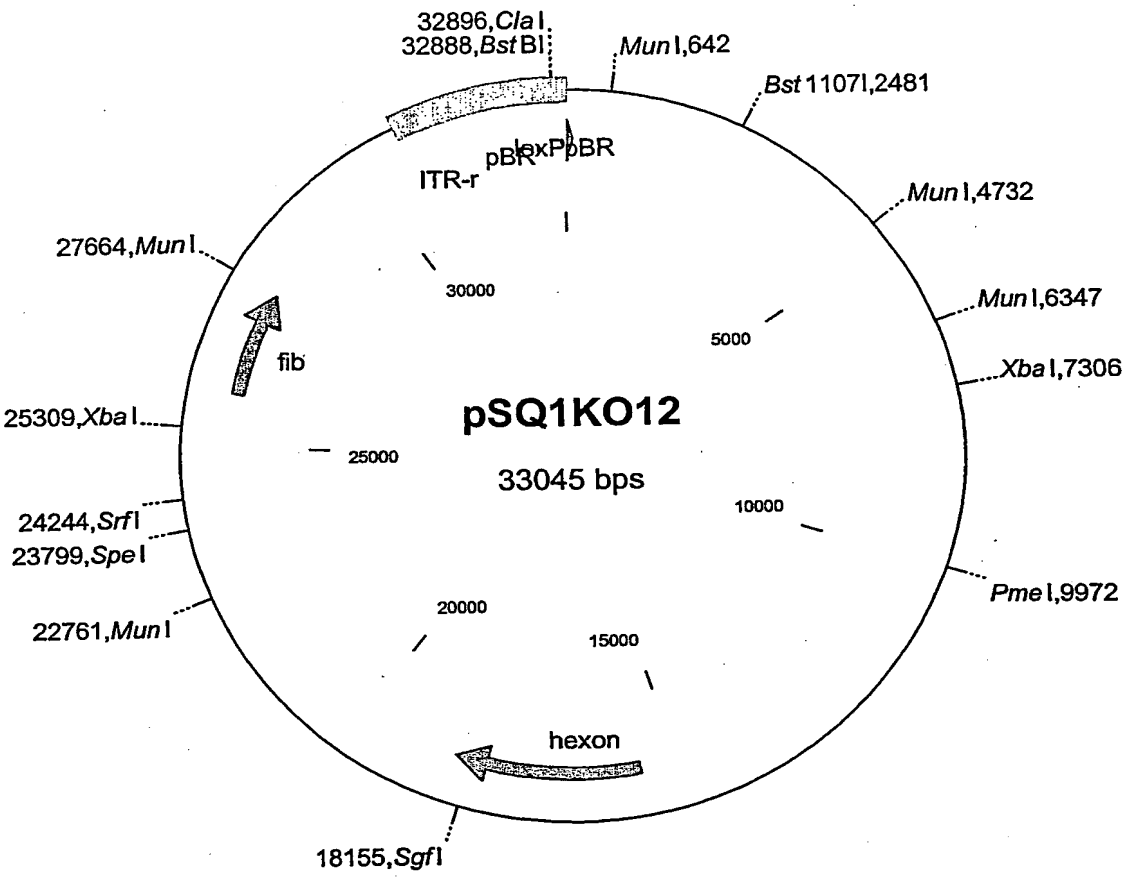

BamHI
ClaI
Csp45I
BstBI
EcoRI
PvuI
NdeI
Bst1107I
PvuI
NotI
PvuI
XbaI
NotI
PvuI
FseI
PmeI
NotI
PvuI
AscI
NotI
NotI
FseI
PvuI
NdeI
BamHI
PvuI
SgfI
PvuI
AscI
SrfI
EcoRI
SpeI
XbaI
NdeI
fib
ITR-r
pBR
loxP
pSQ1
33045 bps
5000
10000
15000
20000
25000
30000
penton
hexon

Figure 3C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32888,BstBI
MunI,642
Bst1107I,2481
MunI,4732
MunI,6347
XbaI,7306
PmeI,9972
18155,SgfI
22761,MunI
23799,SpeI
24244,SrfI
25309,XbaI
27664,MunI
ITR-r
pBR
lexP
pBR
fib
hexon
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10000
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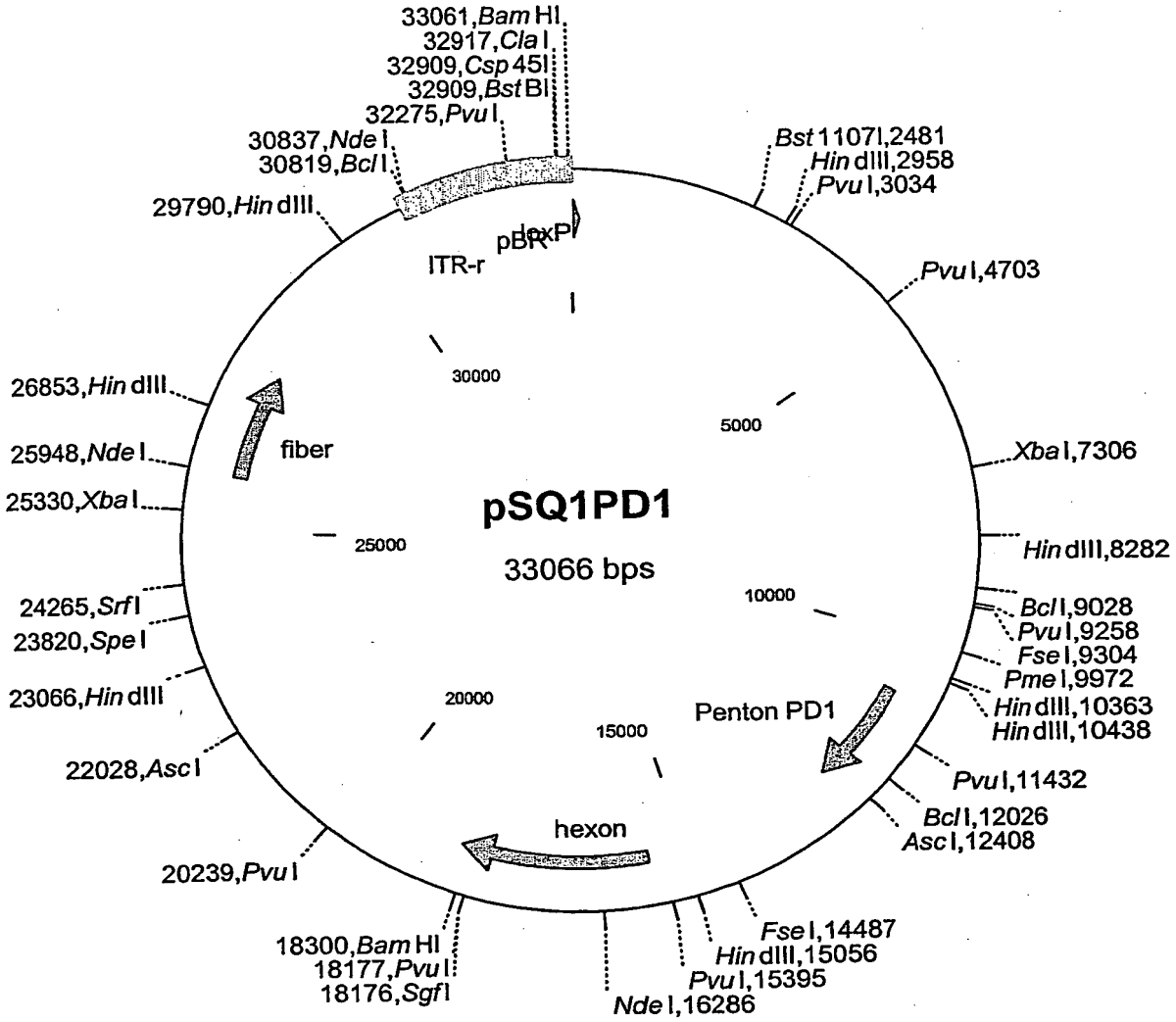
20000
25000
30000

Figure 4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32917,ClaI
32909,Csp45I
32909,BstBI
32275,PvuI
30837,NdeI
30819,BclI
29790,HindIII
ITR-r
pBR
lexP
Bst1107I,2481
HindIII,2958
PvuI,3034
PvuI,4703
XbaI,7306
HindIII,8282
BclI,9028
PvuI,9258
FseI,9304
PmeI,9972
HindIII,10363
HindIII,10438
PvuI,11432
BclI,12026
AscI,12408
FseI,14487
HindIII,15056
PvuI,15395
NdeI,16286
18300,BamHI
18177,PvuI
18176,SgfI
20239,PvuI
22028,AscI
23066,HindIII
23820,SpeI
24265,SrfI
25330,XbaI
25948,NdeI
26853,HindIII
fiber
hexon
Penton PD1
5000
10000
15000
20000
25000
30000

Figure 5A

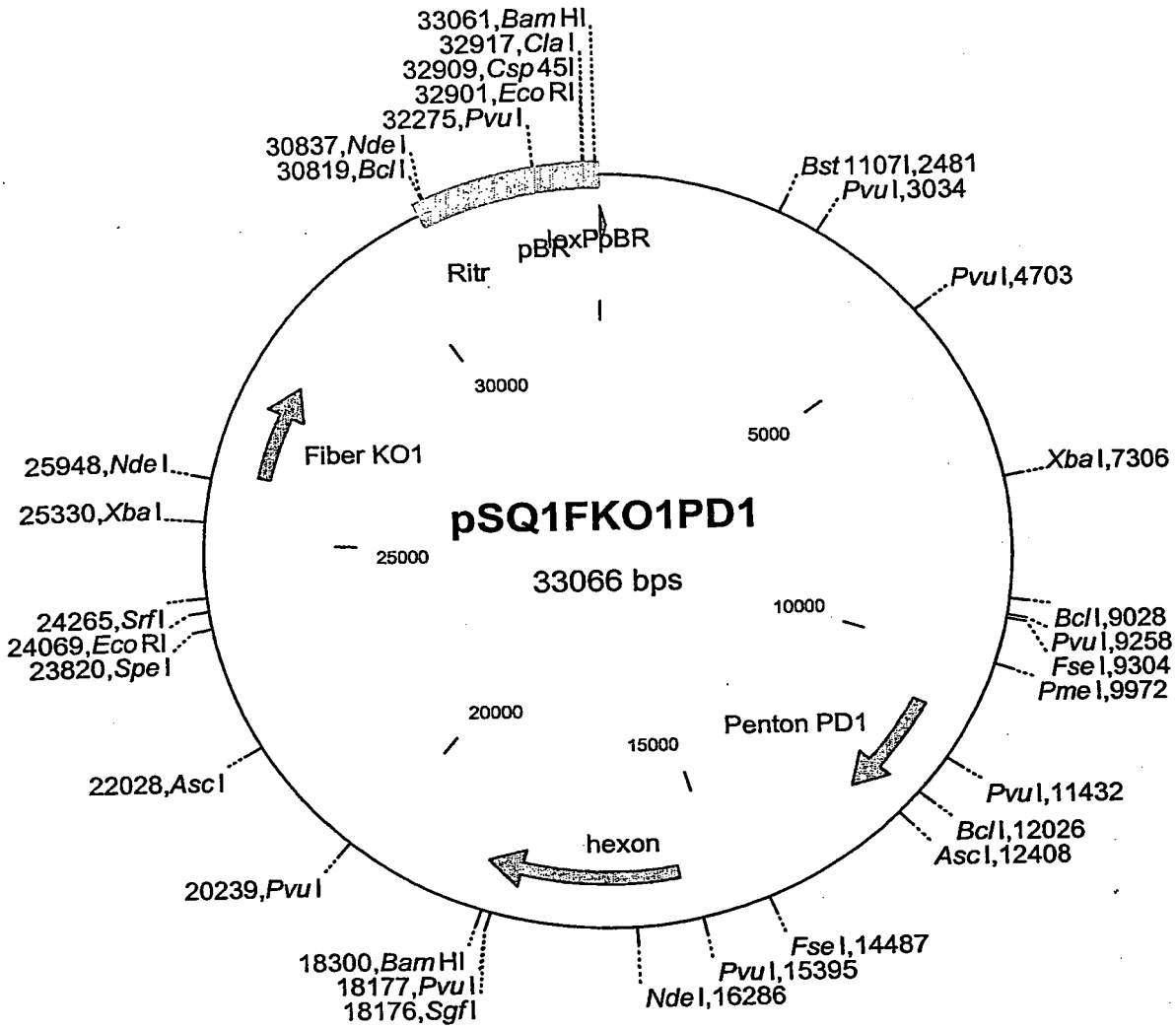
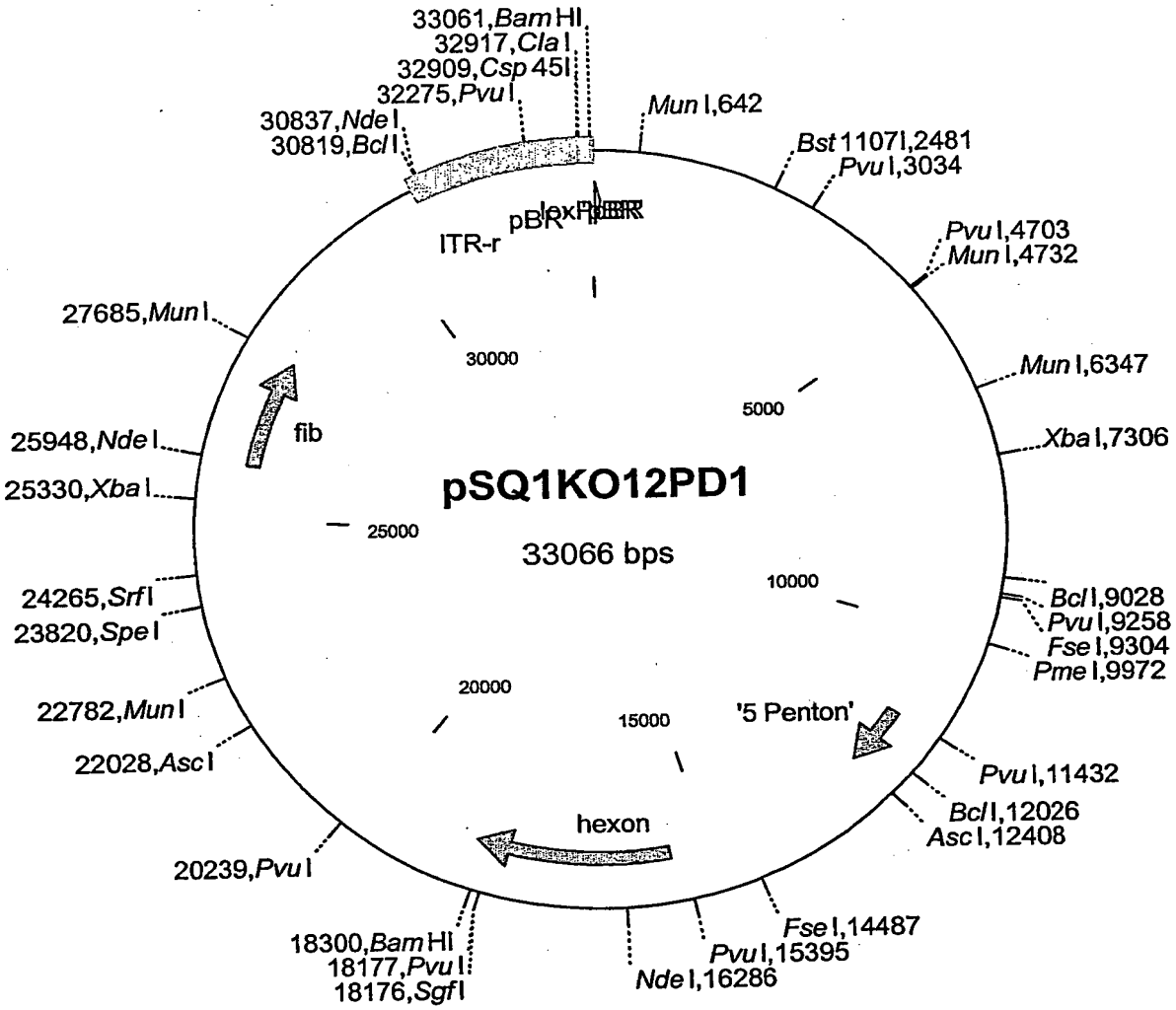

pSQ1FKO1PD1
33066 bps
33061,BamHI
32917,ClaI
32909,Csp45I
32901,EcoRI
32275,PvuI
30837,NdeI
30819,BclI
Bst1107I,2481
PvuI,3034
PvuI,4703
XbaI,7306
BclI,9028
PvuI,9258
FseI,9304
PmeI,9972
PvuI,11432
BclI,12026
AscI,12408
FseI,14487
PvuI,15395
NdeI,16286
18300,BamHI
18177,PvuI
18176,SgfI
20239,PvuI
22028,AscI
24265,SrfI
24069,EcoRI
23820,SpeI
25948,NdeI
25330,XbaI
pBR
lexPpBR
Ritr
Fiber KO1
Penton PD1
hexon
5000
10000
15000
20000
25000
30000

Figure 5B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32917,ClaI
32909,Csp45I
32275,PvuI
30837,NdeI
30819,BclI
MunI,642
BstI1107I,2481
PvuI,3034
PvuI,4703
MunI,4732
MunI,6347
XbaI,7306
BclI,9028
PvuI,9258
FseI,9304
PmeI,9972
PvuI,11432
BclI,12026
AscI,12408
FseI,14487
PvuI,15395
NdeI,16286
18300,BamHI
18177,PvuI
18176,SgfI
20239,PvuI
22028,AscI
22782,MunI
23820,SpeI
24265,SrfI
25330,XbaI
25948,NdeI
27685,MunI
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pBR
lexP
fib
'5 Penton'
hexon
5000
10000
15000
20000
25000
30000

Figure 6

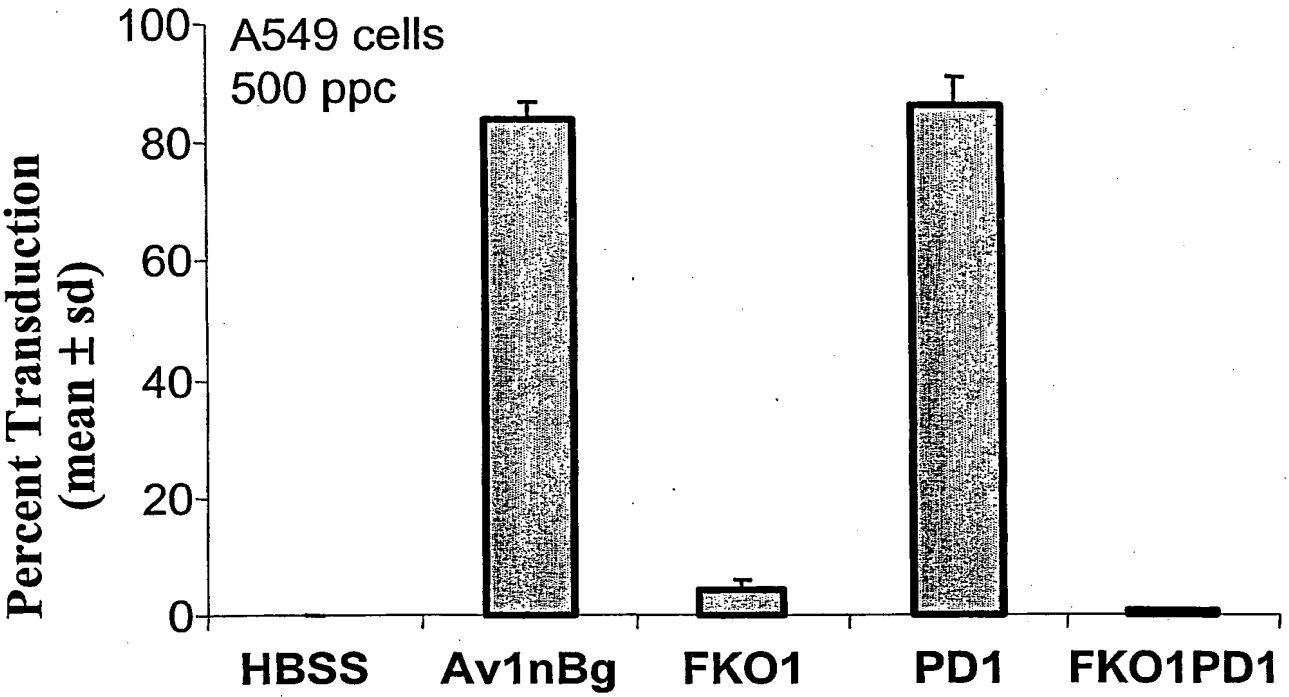

A549 cells
500 ppc
Percent Transduction
(mean ± sd)
100
80
60
40
20
0
HBSS
Av1nBg
FKO1
PD1
FKO1PD1

Figure 7A

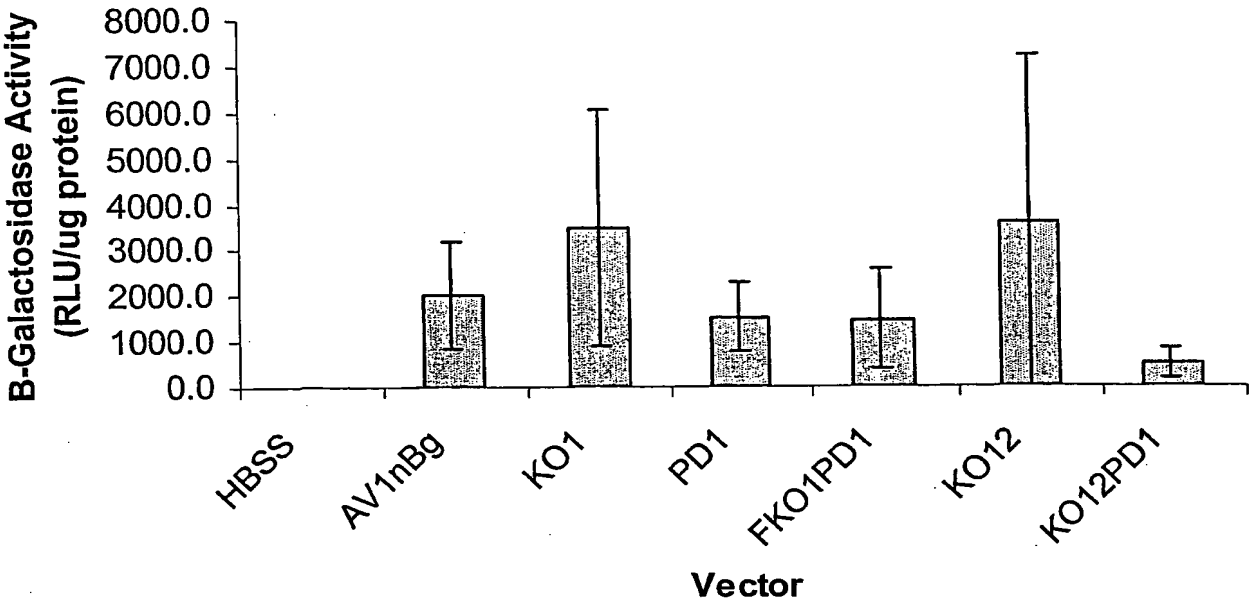


Figure 7B

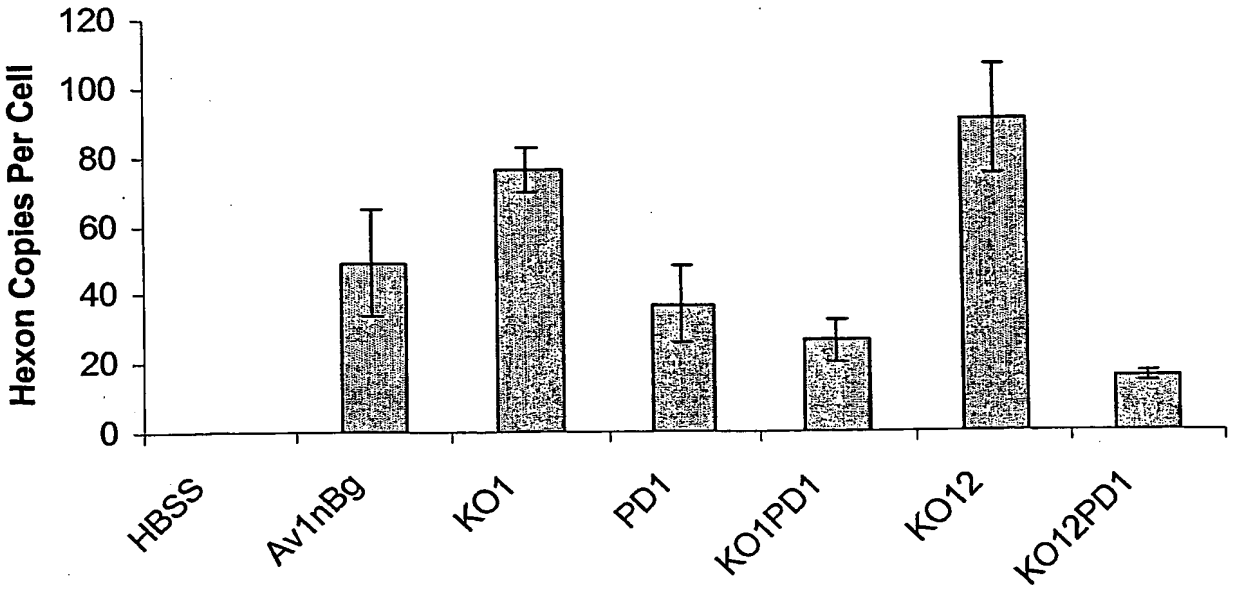


Figure 8

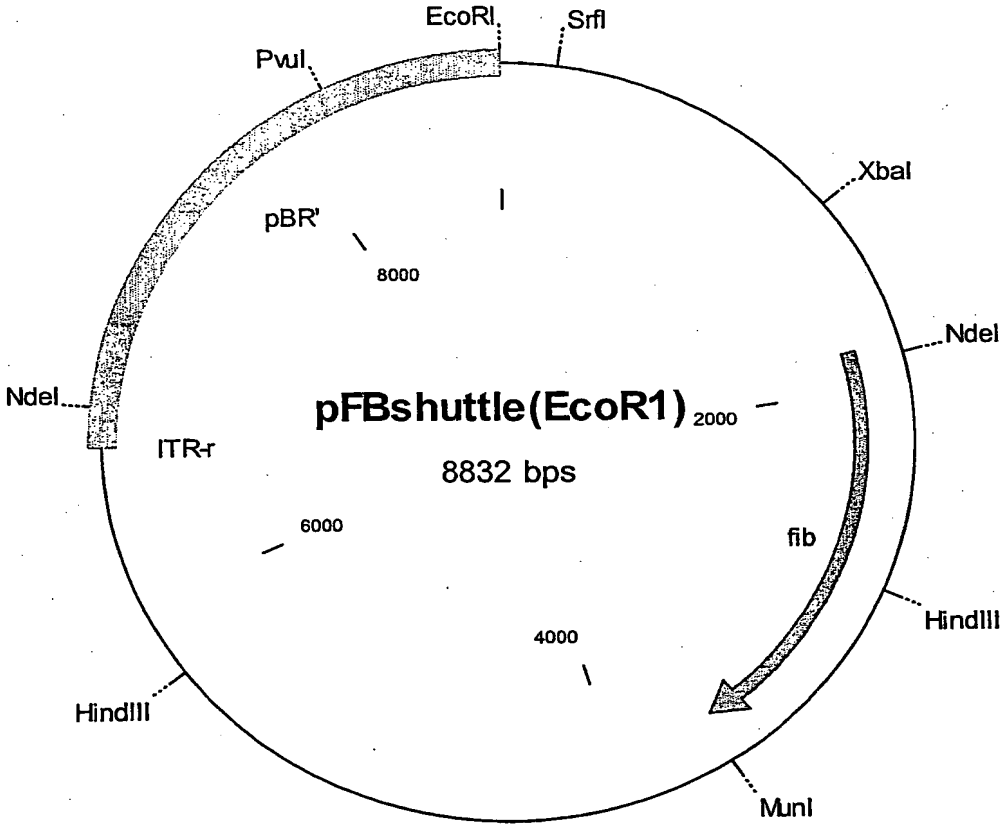
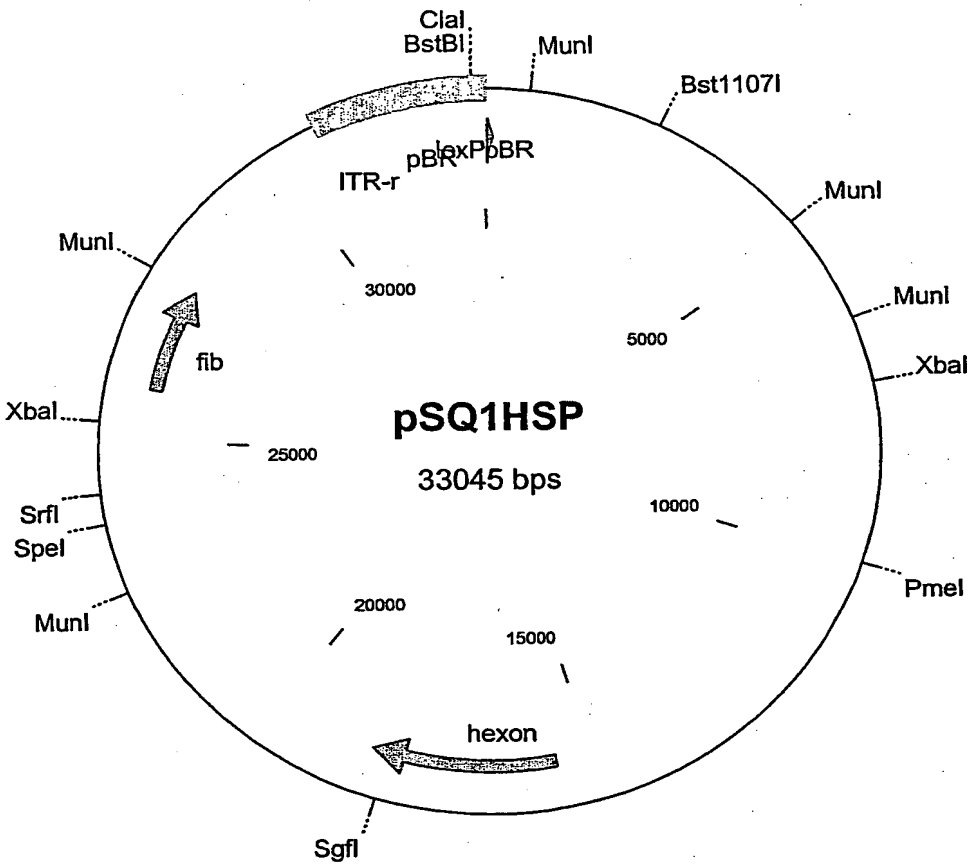

EcoRI
SrfI
PvuI
XbaI
pBR'
8000
NdeI
NdeI
pFBshuttle(EcoR1)
2000
8832 bps
ITR-r
6000
fib
HindIII
4000
HindIII
MunI

Figure 9


ClaI
BstBI
MunI
Bst1107I
MunI
MunI
XbaI
MunI
XbaI
SrfI
SpeI
MunI
PmeI
Sgfl
hexon
fib
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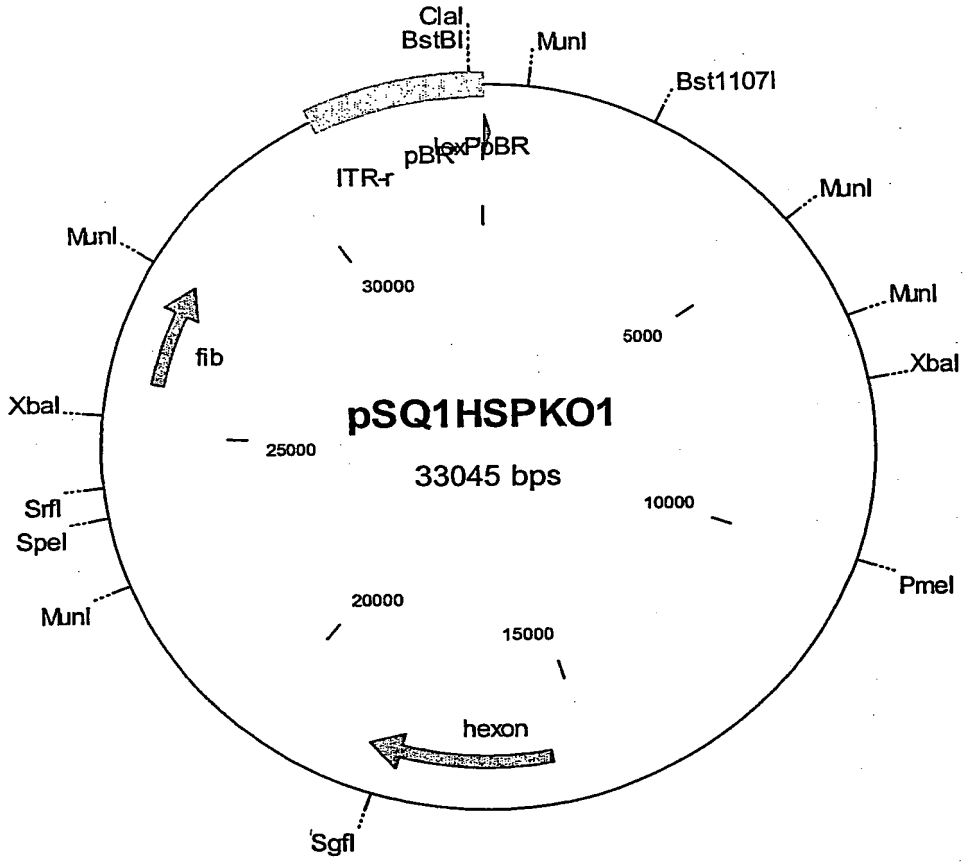
lexP
pBR
pSQ1HSP
33045 bps
5000
10000
15000
20000
25000
30000

Figure 10


ClaI
BstBI
MunI
Bst1107I
MunI
MunI
XbaI
SrfI
SpeI
MunI
PmeI
SgfI
ITR-r
pBR
loxP
pBR
fib
hexon
pSQ1HSPKO1
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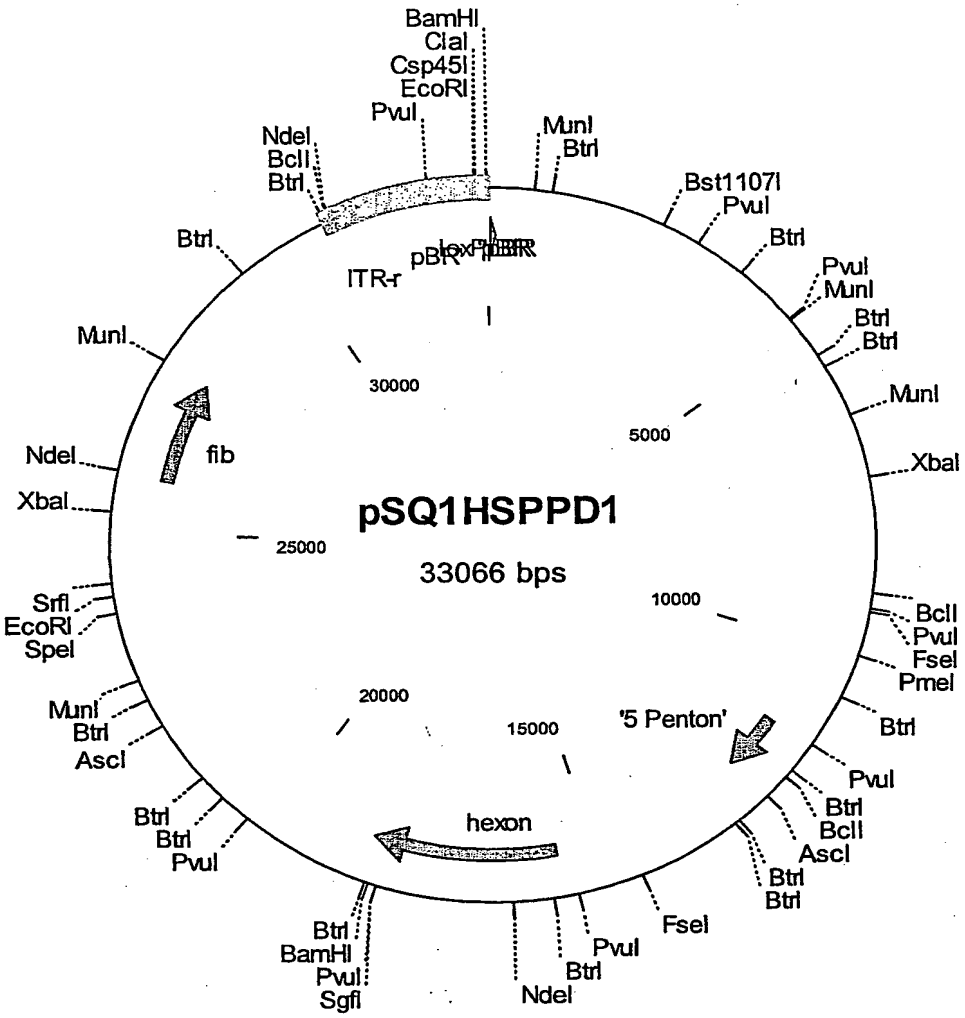
5000
10000
15000
20000
25000
30000

Figure 11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ClaI
Csp45I
EcoRI
PvuI
NdeI
BclI
BtrI
MunI
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Bst1107I
PvuI
BtrI
PvuI
MunI
BtrI
BtrI
MunI
XbaI
BclI
PvuI
FseI
PmeI
BtrI
PvuI
BtrI
BclI
AscI
BtrI
BtrI
FseI
PvuI
BtrI
NdeI
BtrI
PvuI
SgfI
BamHI
BtrI
BtrI
PvuI
BtrI
BtrI
AscI
MunI
BtrI
SrfI
EcoRI
SpeI
NdeI
XbaI
MunI
BtrI
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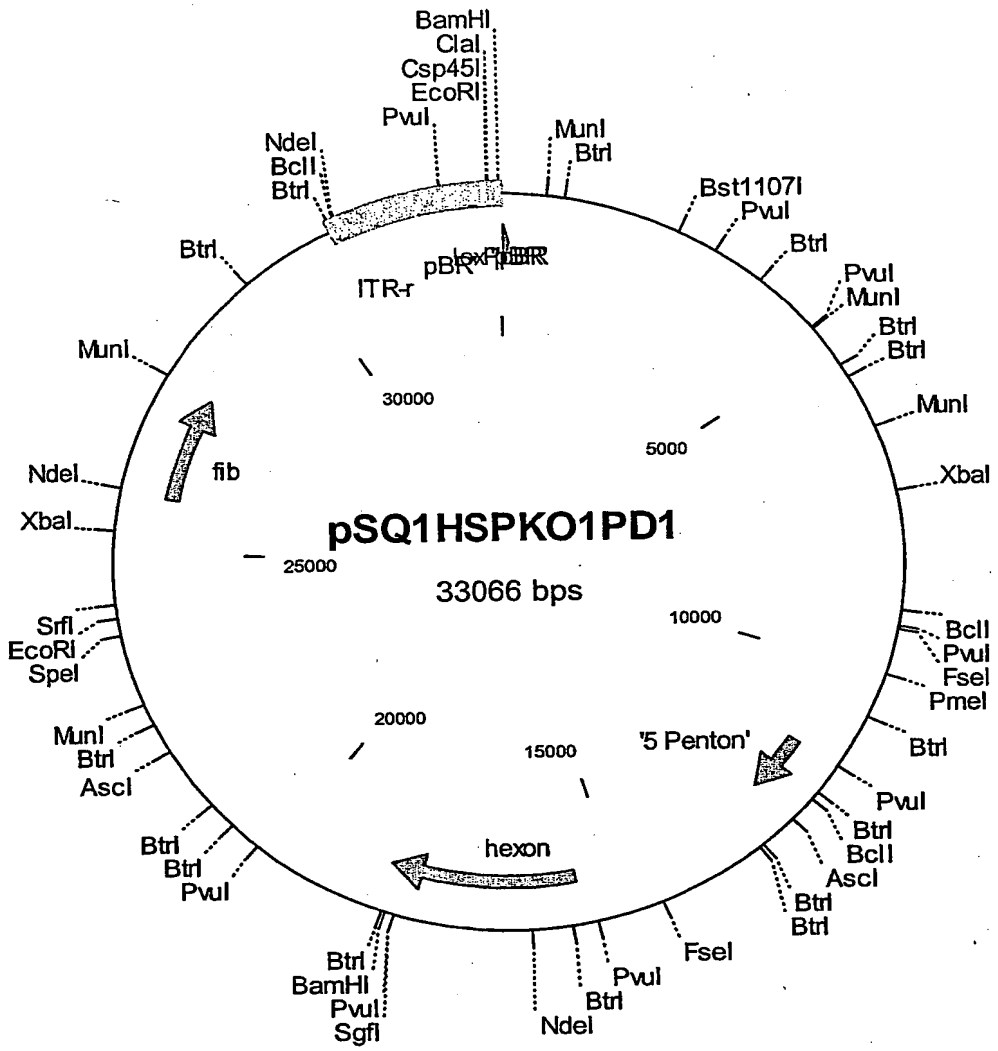
pBR
fib
hexon
'5 Penton'
5000
10000
15000
20000
25000
30000

Figure 12


pSQ1HSPKO1PD1
33066 bps
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10000
15000
20000
25000
30000
ITR-r
pBR
fib
hexon
'5 Penton'
BamHI
ClaI
Csp45I
EcoRI
PvuI
NdeI
BclI
BtrI
MunI
Bst1107I
XbaI
FseI
PmeI
AscI
SrfI
SpeI
SgfI

Figure 13A

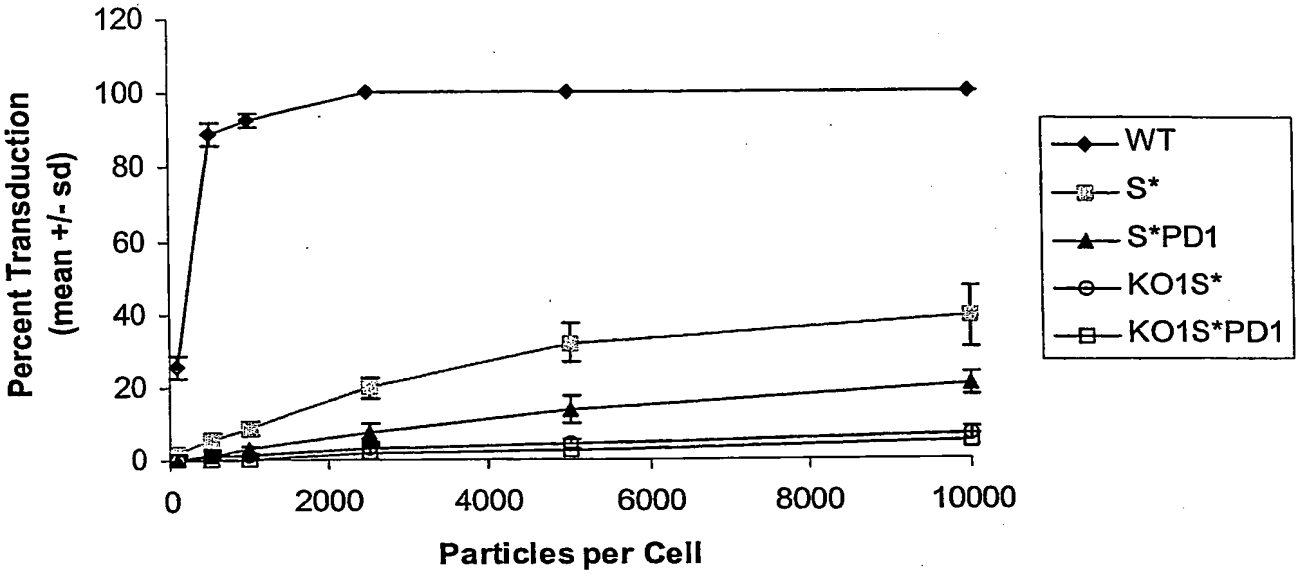


Figure 13B

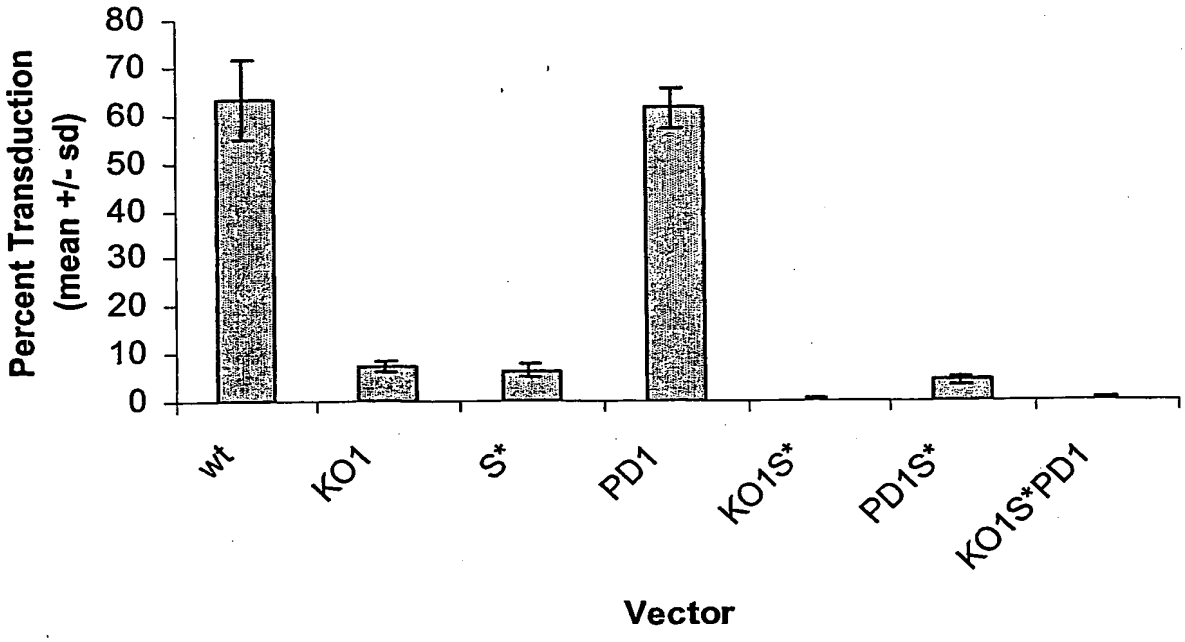


Figure 13C

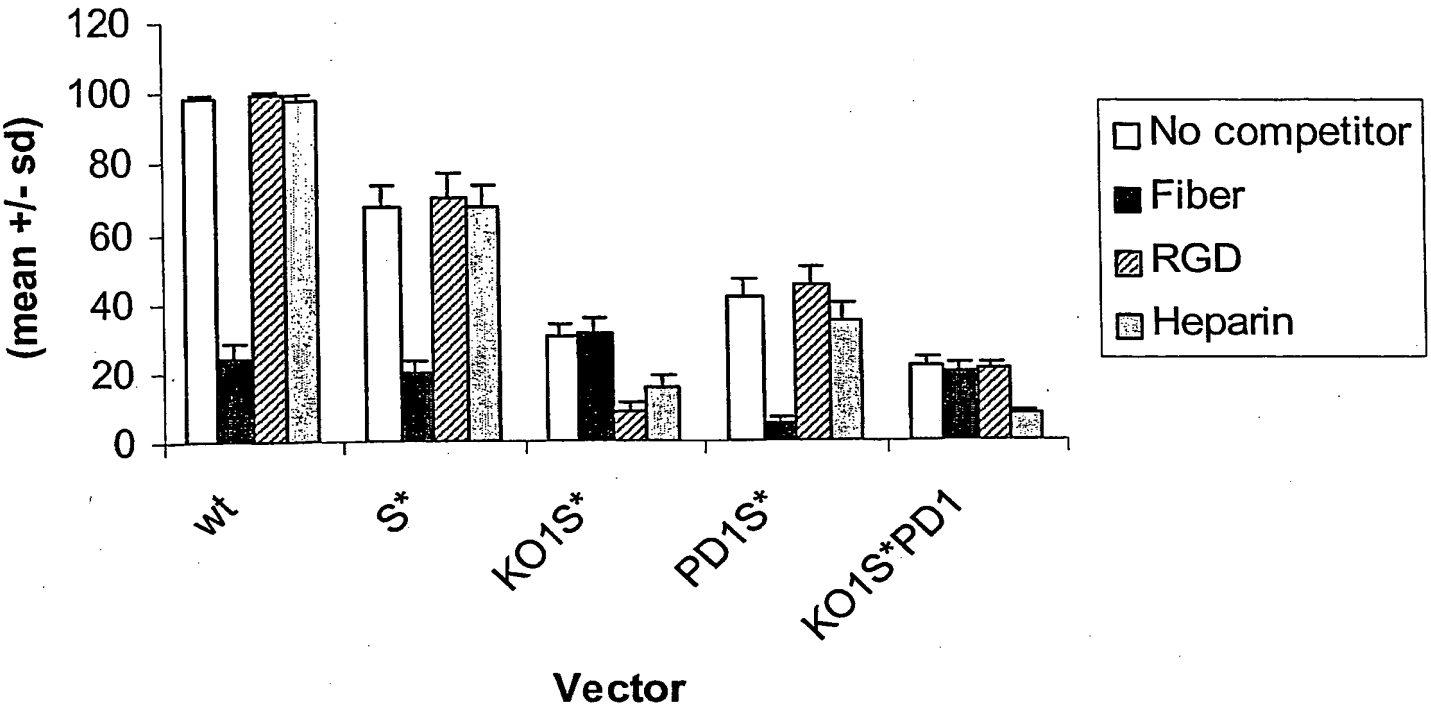

120
100
80
60
40
20
0
(mean +/- sd)
wt
S*
KO1S*
PD1S*
KO1S*PD1
Vector
No competitor
Fiber
RGD
Heparin

Figure 14A

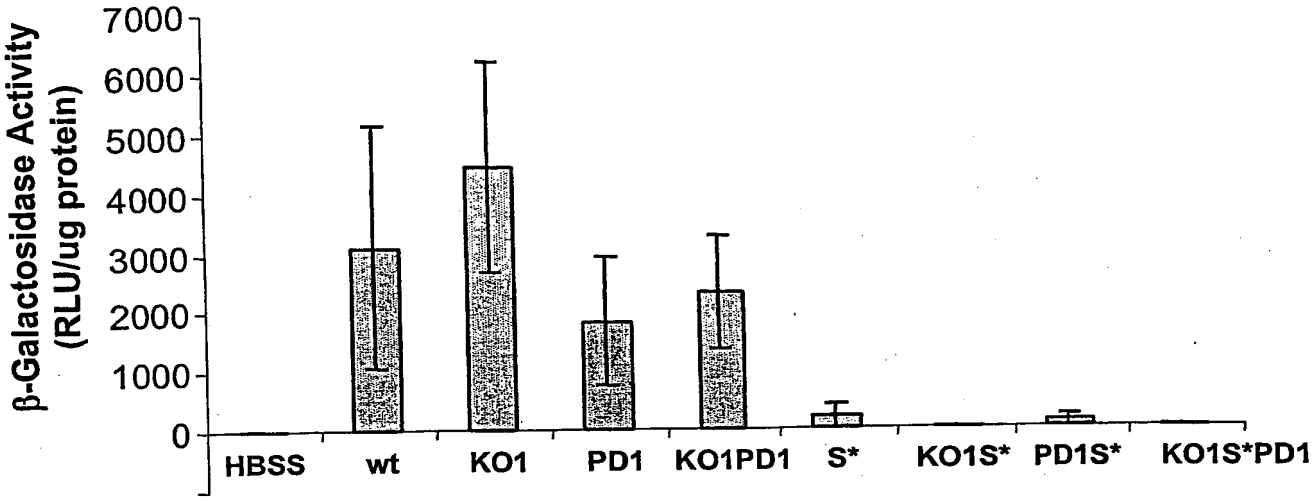


Figure 14B

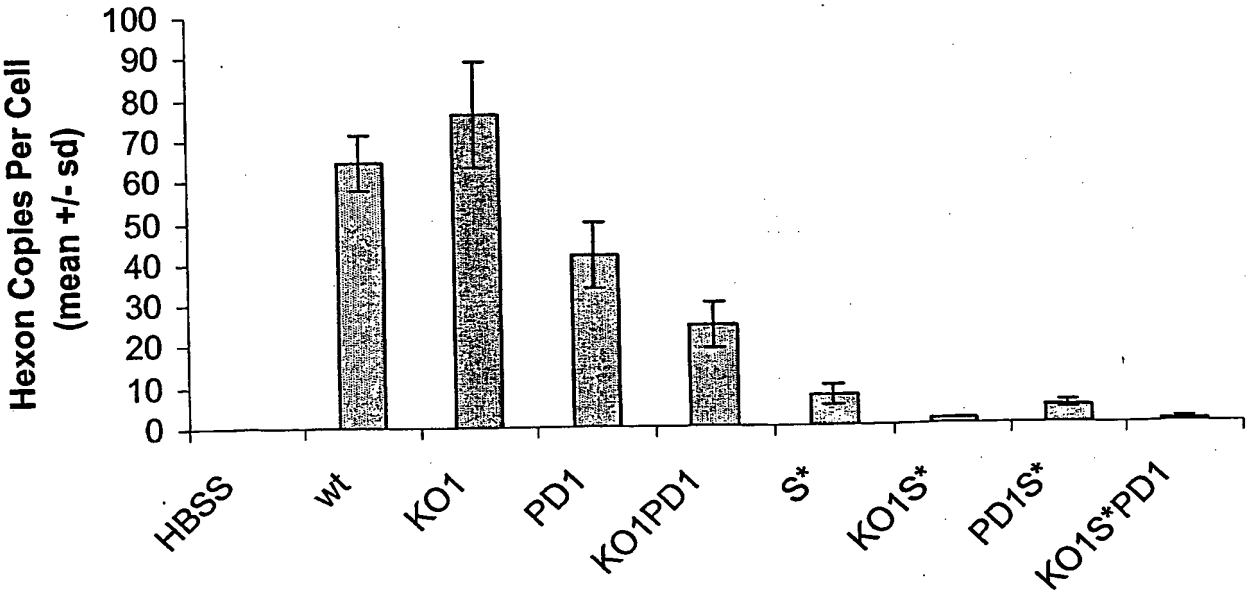


Figure 15A

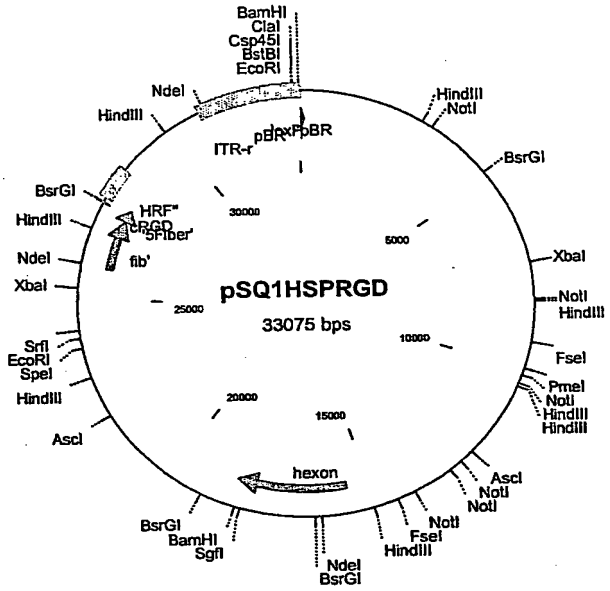

BamHI
ClaI
Csp45I
BstBI
EcoRI
NdeI
HindIII
HindIII
NotI
BsrGI
pBR
pBR
ITR-r
BsrGI
HindIII
HRF"
RGD
5'Fiber'
fib'
NdeI
XbaI
pSQ1HSPRGD
33075 bps
30000
5000
25000
10000
20000
15000
XbaI
NotI
HindIII
FseI
PmeI
NotI
HindIII
HindIII
SrfI
EcoRI
SpeI
HindIII
AscI
hexon
AscI
NotI
NotI
NotI
FseI
HindIII
BsrGI
BamHI
SgfI
NdeI
BsrGI

Figure 15B

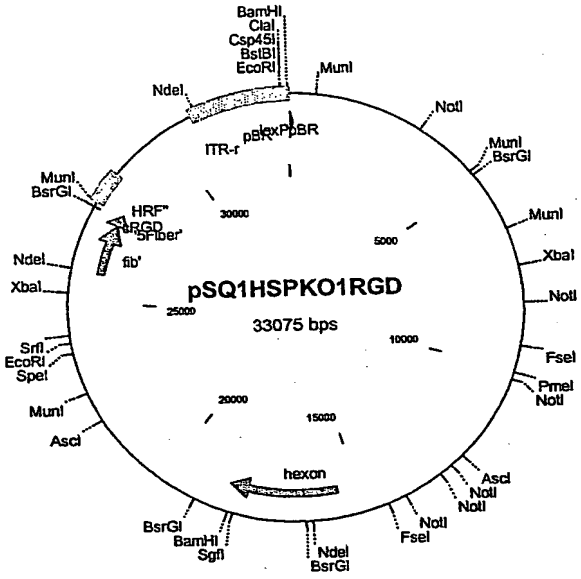

pSQ1HSPKO1RGD
33075 bps
BamHI
ClaI
Csp45I
BstBI
EcoRI
MunI
NotI
MunI
BsrGI
MunI
XbaI
NotI
FseI
PmeI
NotI
AscI
NotI
NotI
NotI
FseI
NdeI
BsrGI
BsrGI
BamHI
SgfI
hexon
AscI
MunI
SrfI
EcoRI
SpeI
XbaI
NdeI
MunI
BsrGI
NdeI
pBR
pBR
ITR-r
HRF"
RGD
5'fiber'
fib'
5000
10000
15000
20000
25000
30000

Figure 16

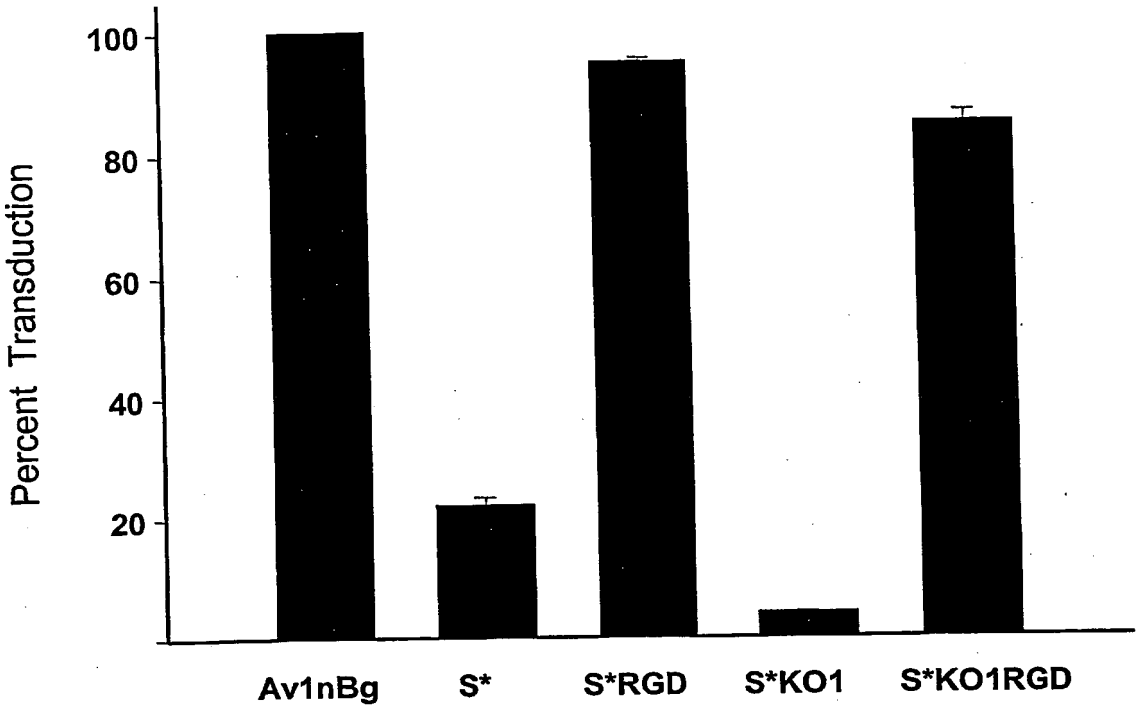

Percent Transduction
100
80
60
40
20
Av1nBg
S*
S*RGD
S*KO1
S*KO1RGD

Figure 17A

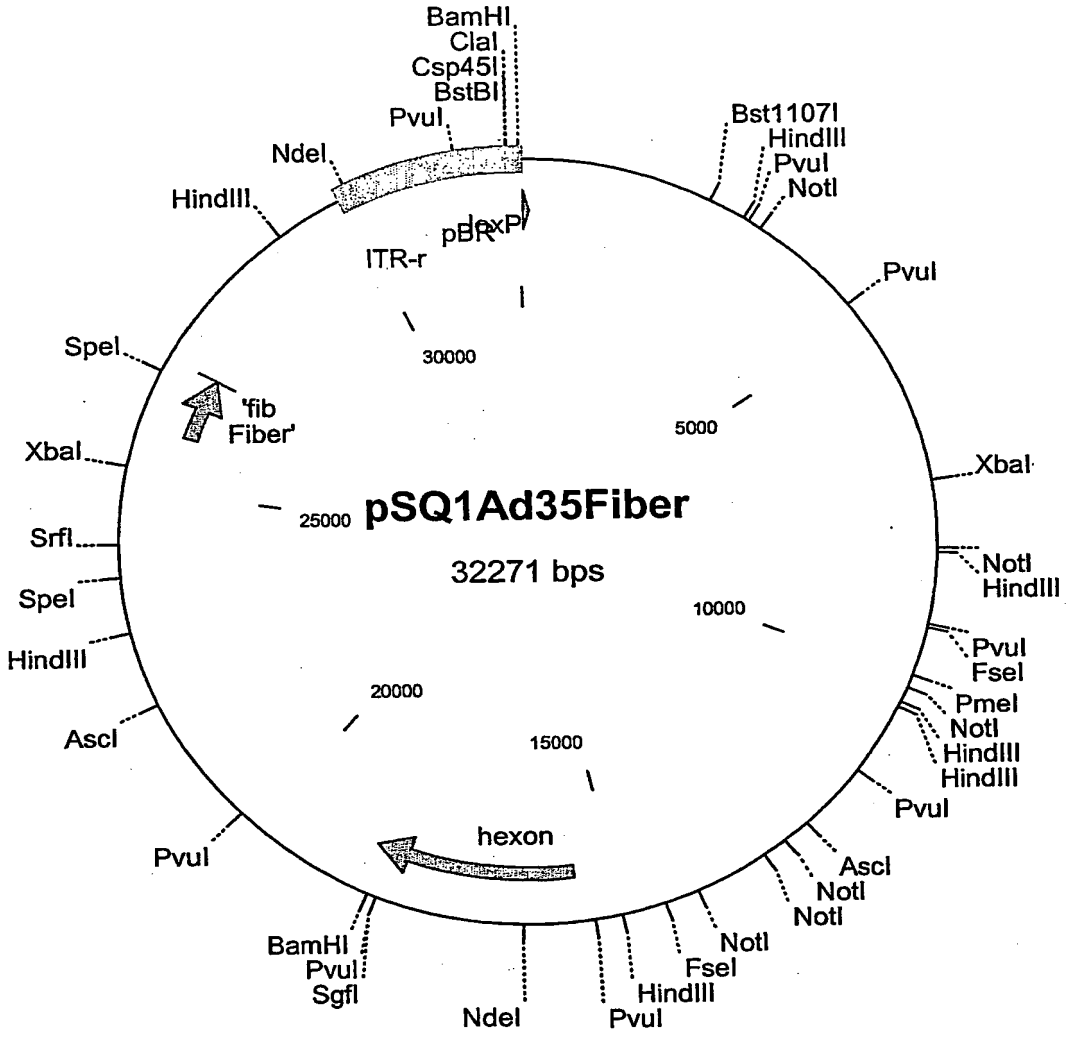

BamHI
ClaI
Csp45I
BstBI
PvuI
NdeI
HindIII
pBR
ITR-r
SpeI
'fib
Fiber'
XbaI
SrfI
SpeI
HindIII
AscI
PvuI
BamHI
PvuI
SgfI
NdeI
PvuI
HindIII
FseI
NotI
AscI
NotI
NotI
PvuI
HindIII
HindIII
NotI
PmeI
FseI
PvuI
NotI
HindIII
XbaI
PvuI
Bst1107I
HindIII
PvuI
NotI
pSQ1Ad35Fiber
32271 bps
5000
10000
15000
20000
25000
30000
hexon

Figure 17B

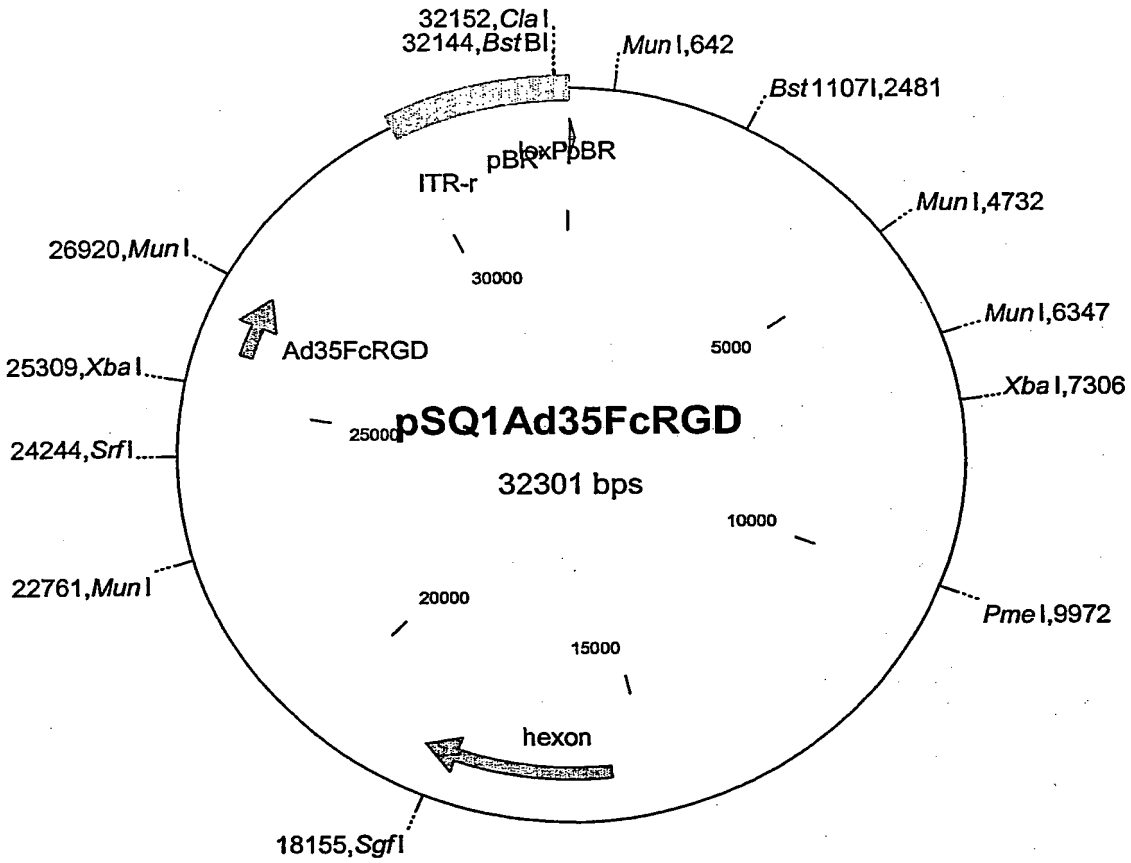

32152,ClaI
32144,BstBI
MunI,642
Bst1107I,2481
MunI,4732
MunI,6347
XbaI,7306
PmeI,9972
18155,SgfI
hexon
22761,MunI
24244,SrfI
25309,XbaI
26920,MunI
ITR-r
pBR
loxP
pBR
Ad35FcRGD
pSQ1Ad35FcRGD
32301 bps
5000
10000
15000
20000
25000
30000

Figure 18A

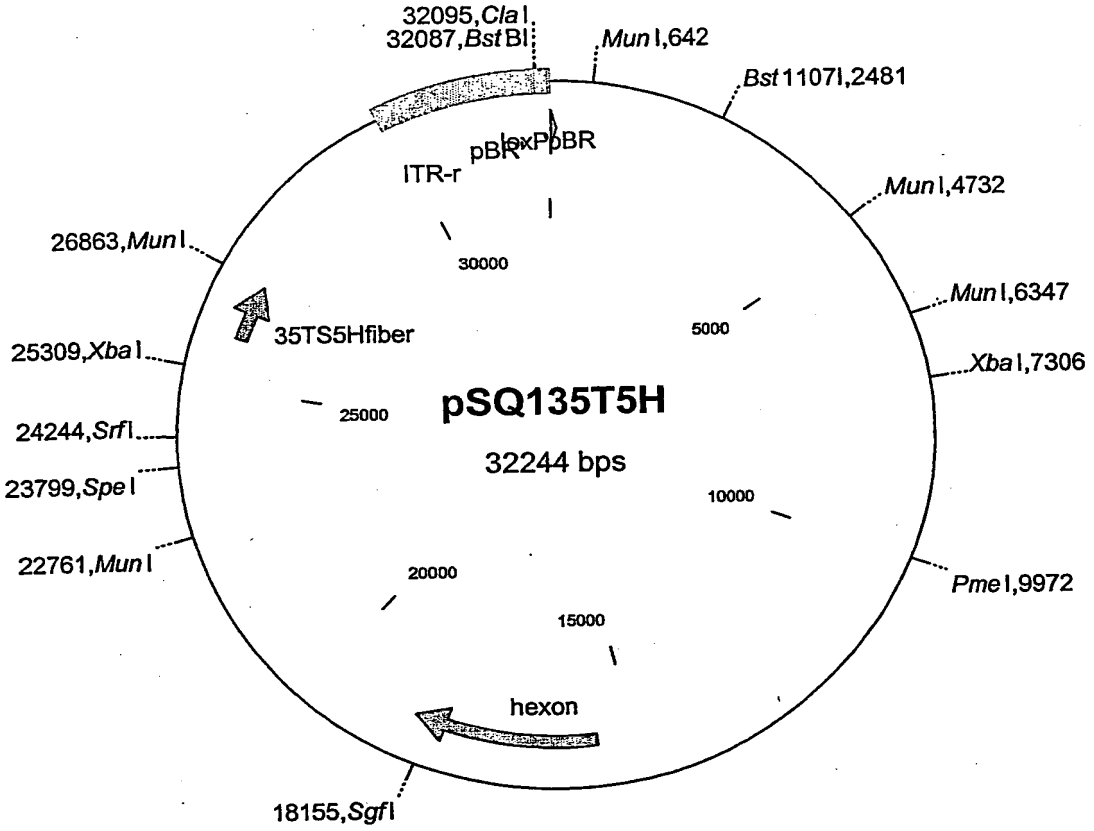

32095,ClaI
32087,BstBI
MunI,642
Bst1107I,2481
MunI,4732
MunI,6347
XbaI,7306
PmeI,9972
18155,SgfI
hexon
ITR-r
pBR
lexPpBR
35TS5Hfiber
26863,MunI
25309,XbaI
24244,SrfI
23799,SpeI
22761,MunI
pSQ135T5H
32244 bps
5000
10000
15000
20000
25000
30000

Figure 18B

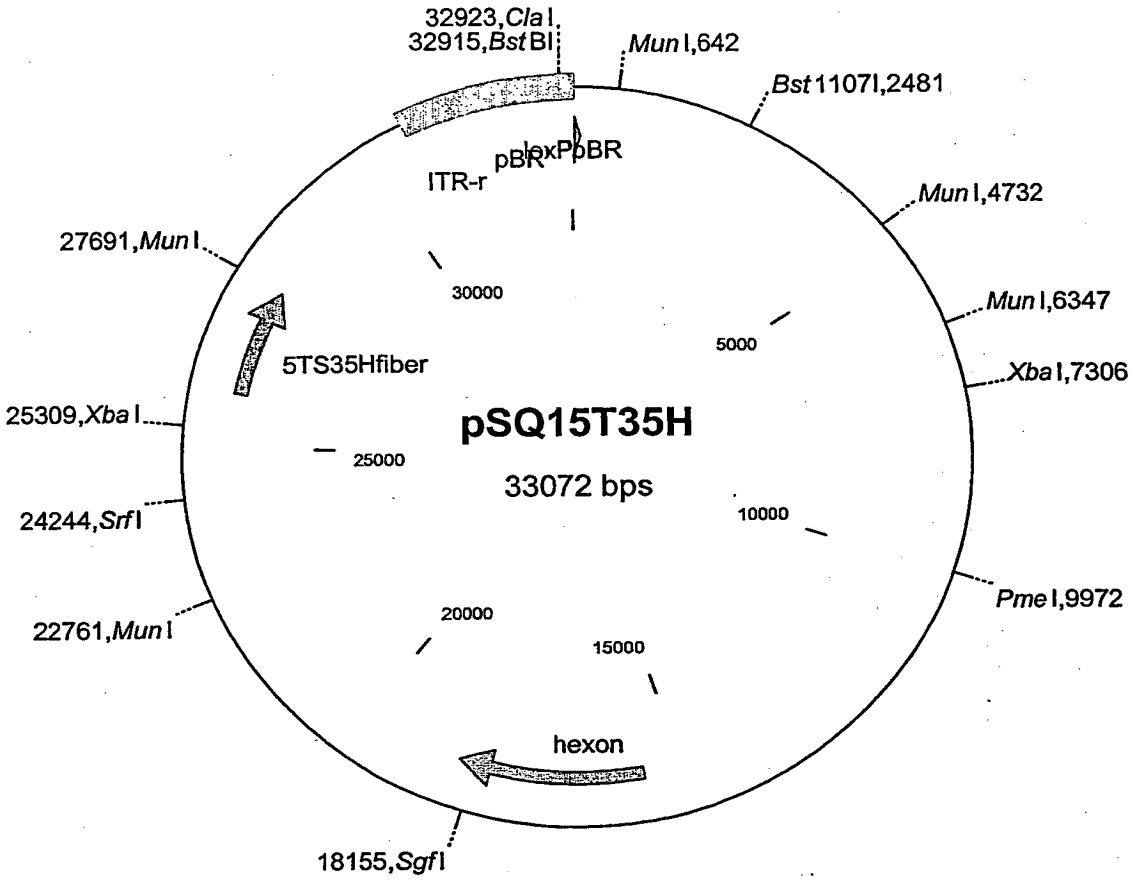

32923,Cla I
32915,Bst BI
Mun I,642
Bst 1107I,2481
Mun I,4732
Mun I,6347
Xba I,7306
Pme I,9972
18155,Sgf I
22761,Mun I
24244,Srf I
25309,Xba I
27691,Mun I
ITR-r
pBR
loxP
pBR
5TS35Hfiber
hexon
pSQ15T35H
33072 bps
5000
10000
15000
20000
25000
30000

Figure 19

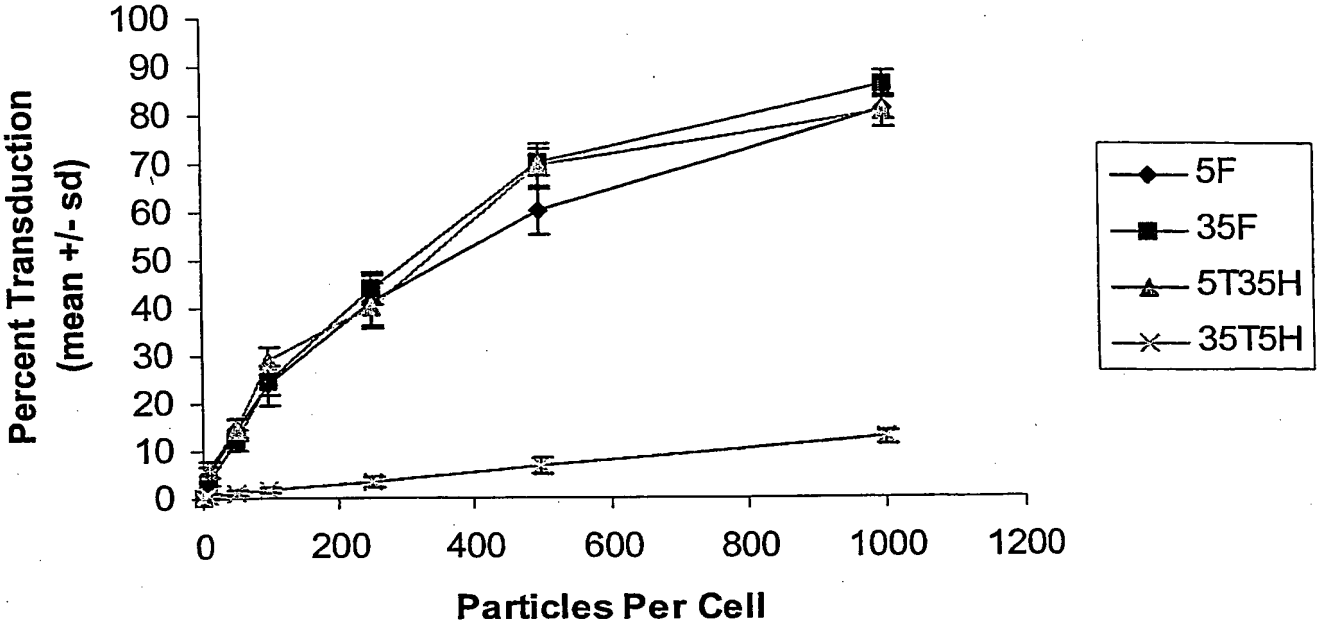

Percent Transduction
(mean +/- sd)
100
90
80
70
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
Particles Per Cell
5F
35F
5T35H
35T5H

Figure 20

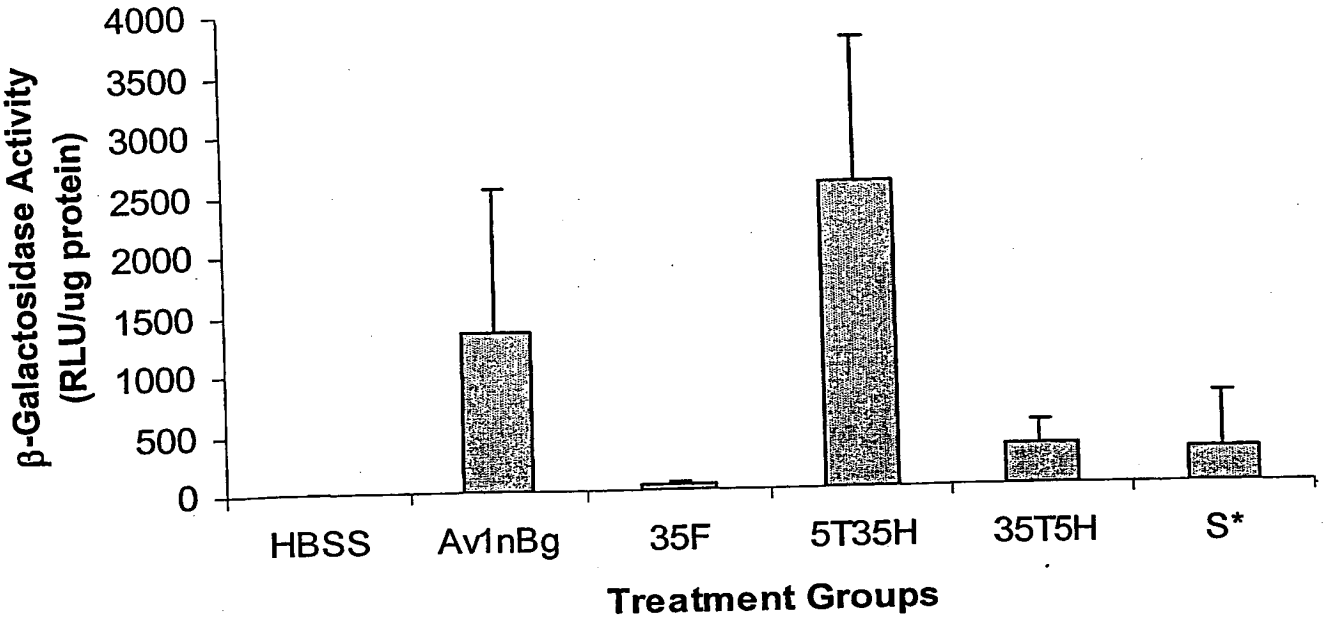

β-Galactosidase Activity (RLU/ug protein)
4000
3500
3000
2500
2000
1500
1000
500
0
HBSS
Av1nBg
35F
5T35H
35T5H
S*
Treatment Groups

Figure 21A

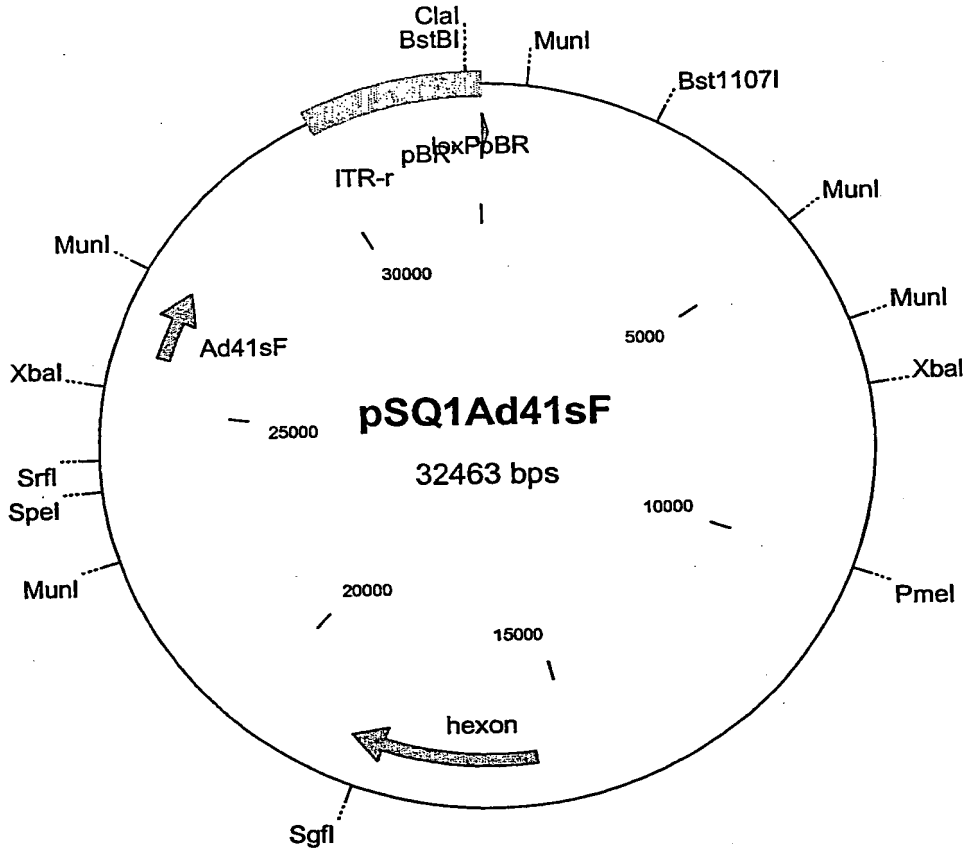
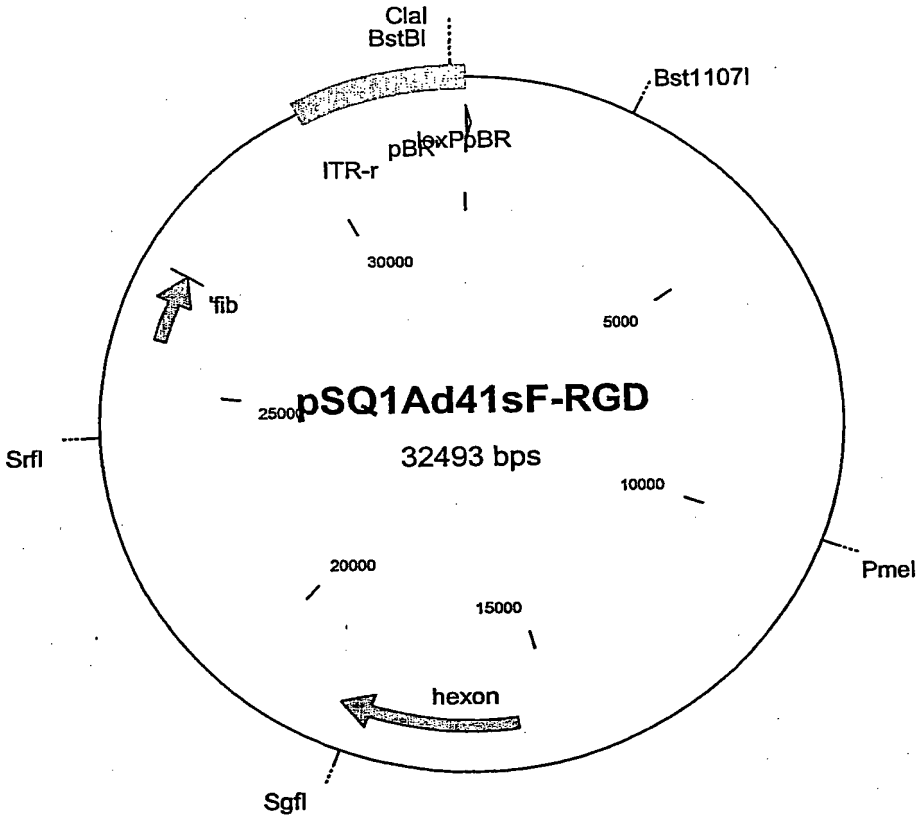

ClaI
BstBI
MunI
Bst1107I
pBR
loxP
pBR
ITR-r
MunI
MunI
MunI
30000
5000
Ad41sF
XbaI
XbaI
pSQ1Ad41sF
25000
32463 bps
SrfI
SpeI
10000
MunI
PmeI
20000
15000
hexon
SgfI

Figure 21B


ClaI
BstBI
Bst1107I
pBR
loxP
pBR
ITR-r
30000
5000
'fib
pSQ1Ad41sF-RGD
25000
32493 bps
SrfI
10000
PmeI
20000
15000
hexon
SgfI

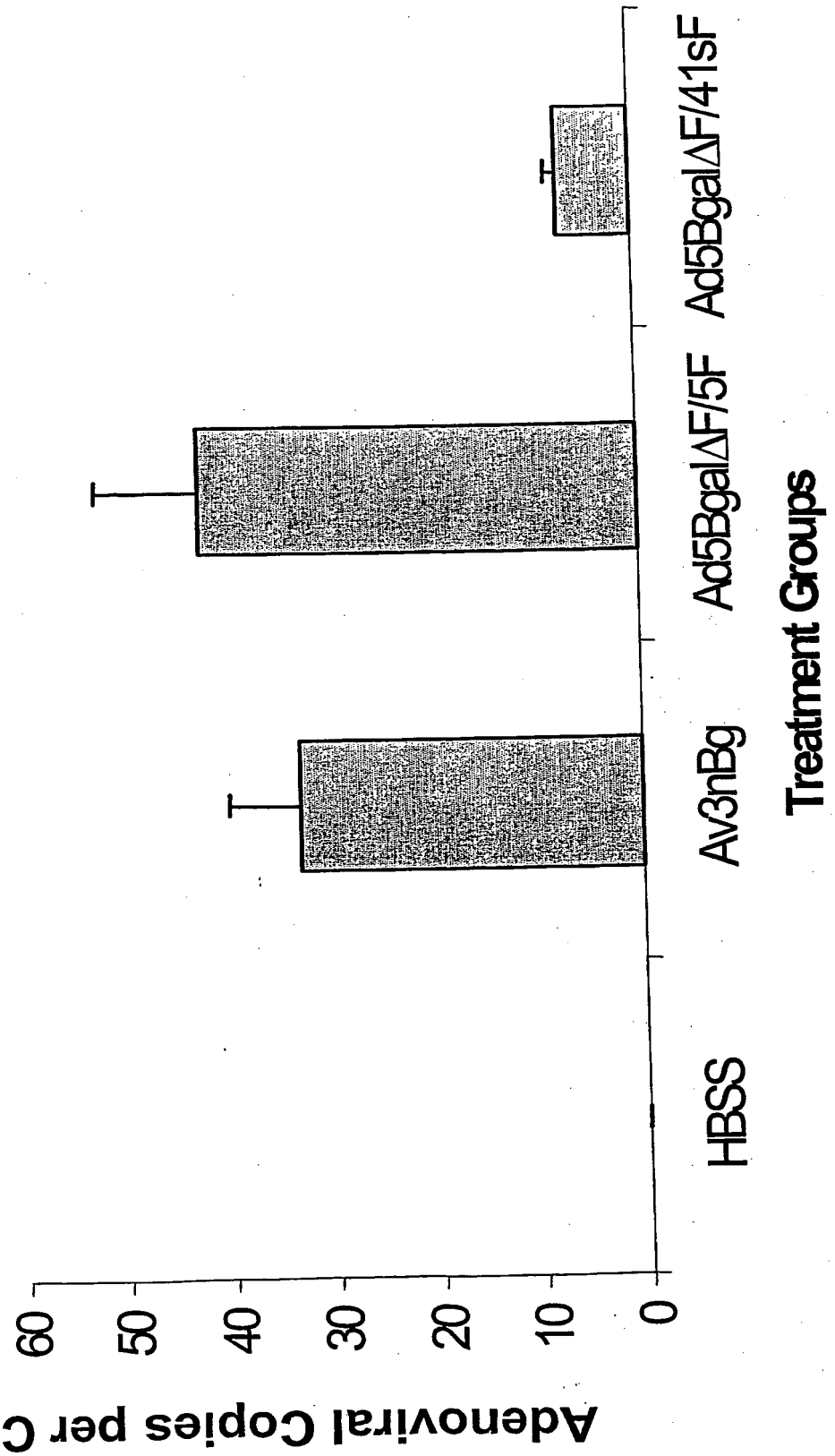

Adenoviral Copies per Cell
0
10
20
30
40
50
60
HBSS
Av3nBg
Ad5BgalΔF/5F
Ad5BgalΔF/41sF
Treatment Groups

Figure 22

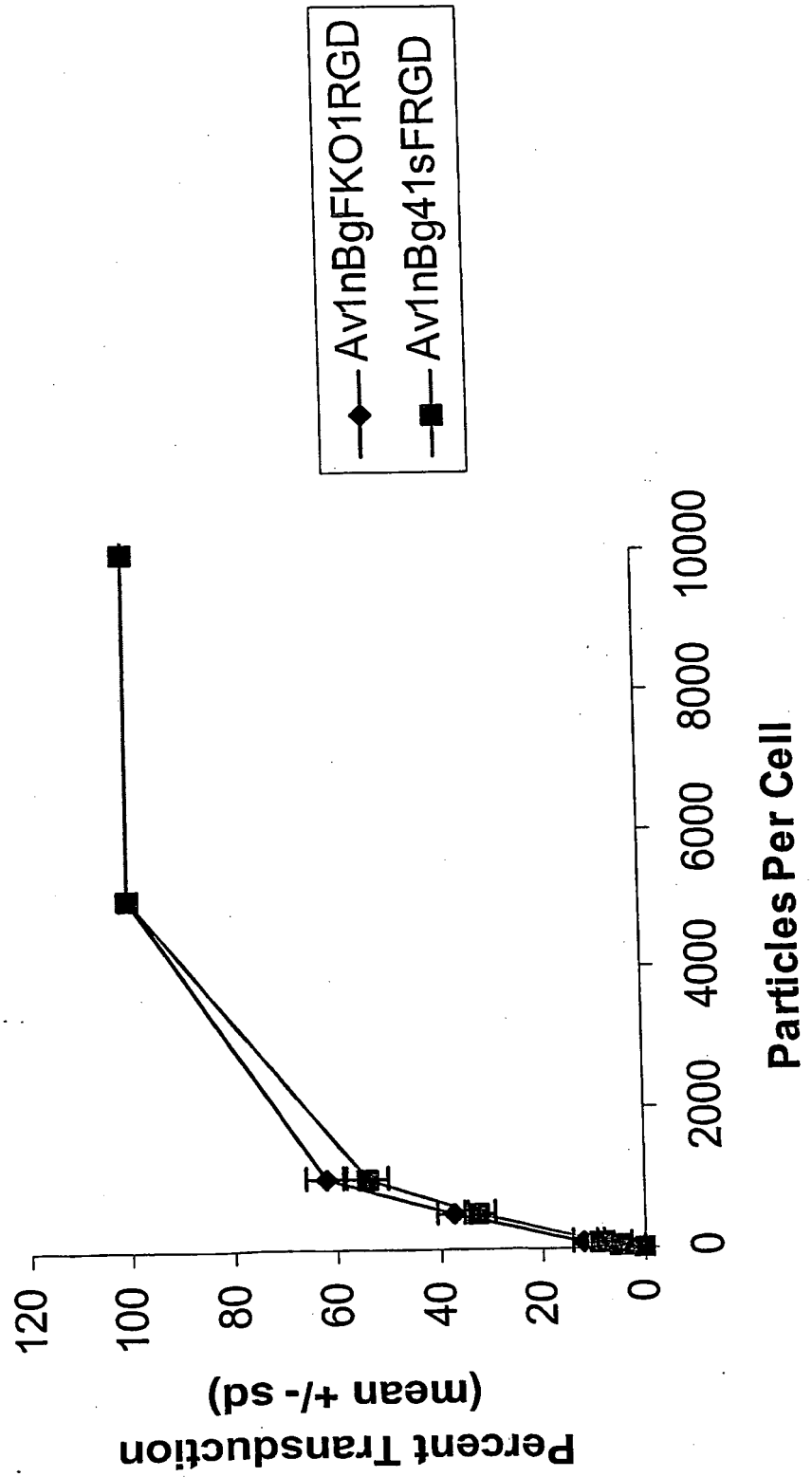

Av1nBgFKO1RGD
Av1nBg41sFRGD
Percent Transduction
(mean +/- sd)
120
100
80
60
40
20
0
0
2000
4000
6000
8000
10000
Particles Per Cell

Figure 23

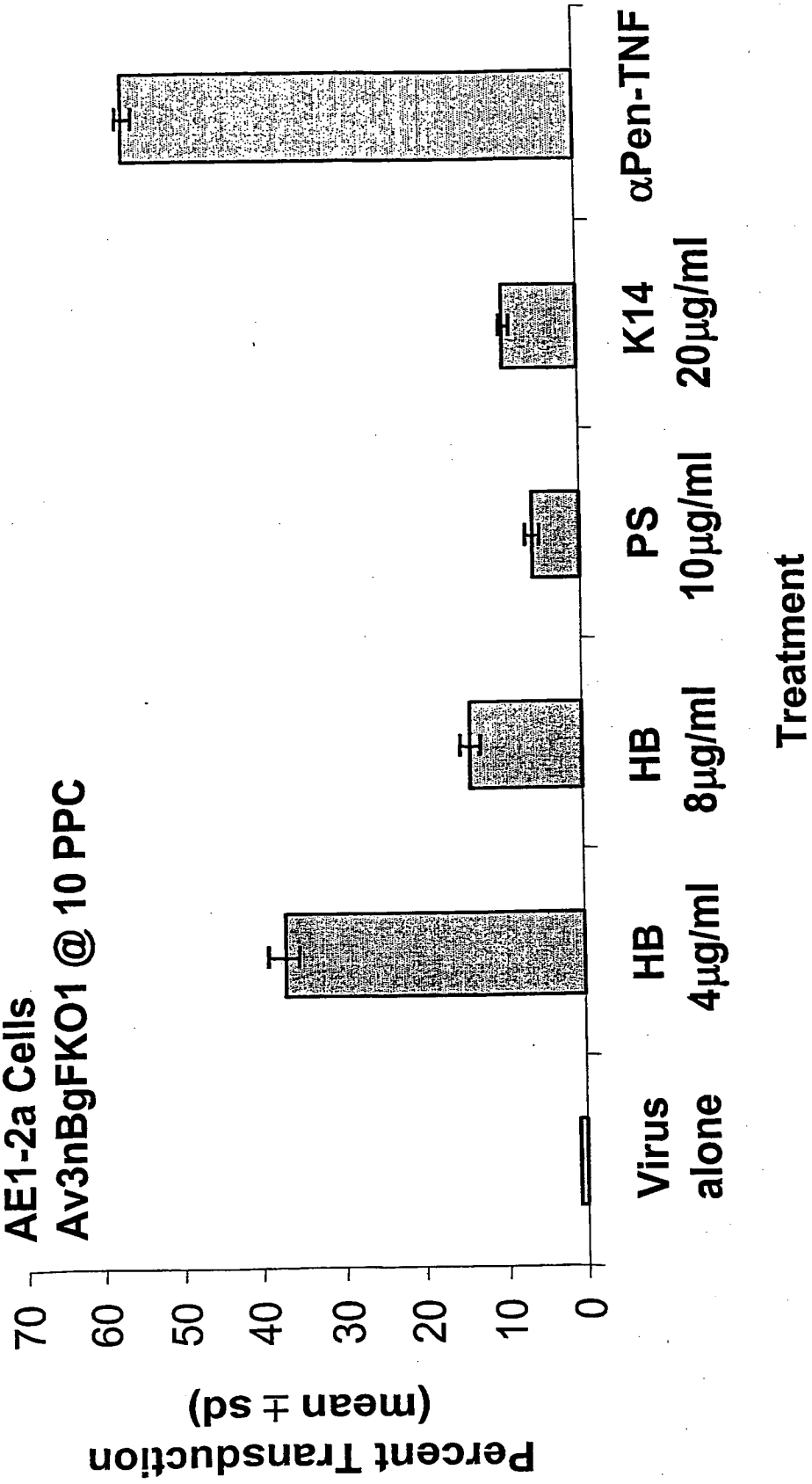

AE1-2a Cells
Av3nBgFKO1 @ 10 PPC
Percent Transduction
(mean ± sd)
70
60
50
40
30
20
10
0
Virus alone
HB 4µg/ml
HB 8µg/ml
PS 10µg/ml
K14 20µg/ml
αPen-TNF
Treatment

Figure 24

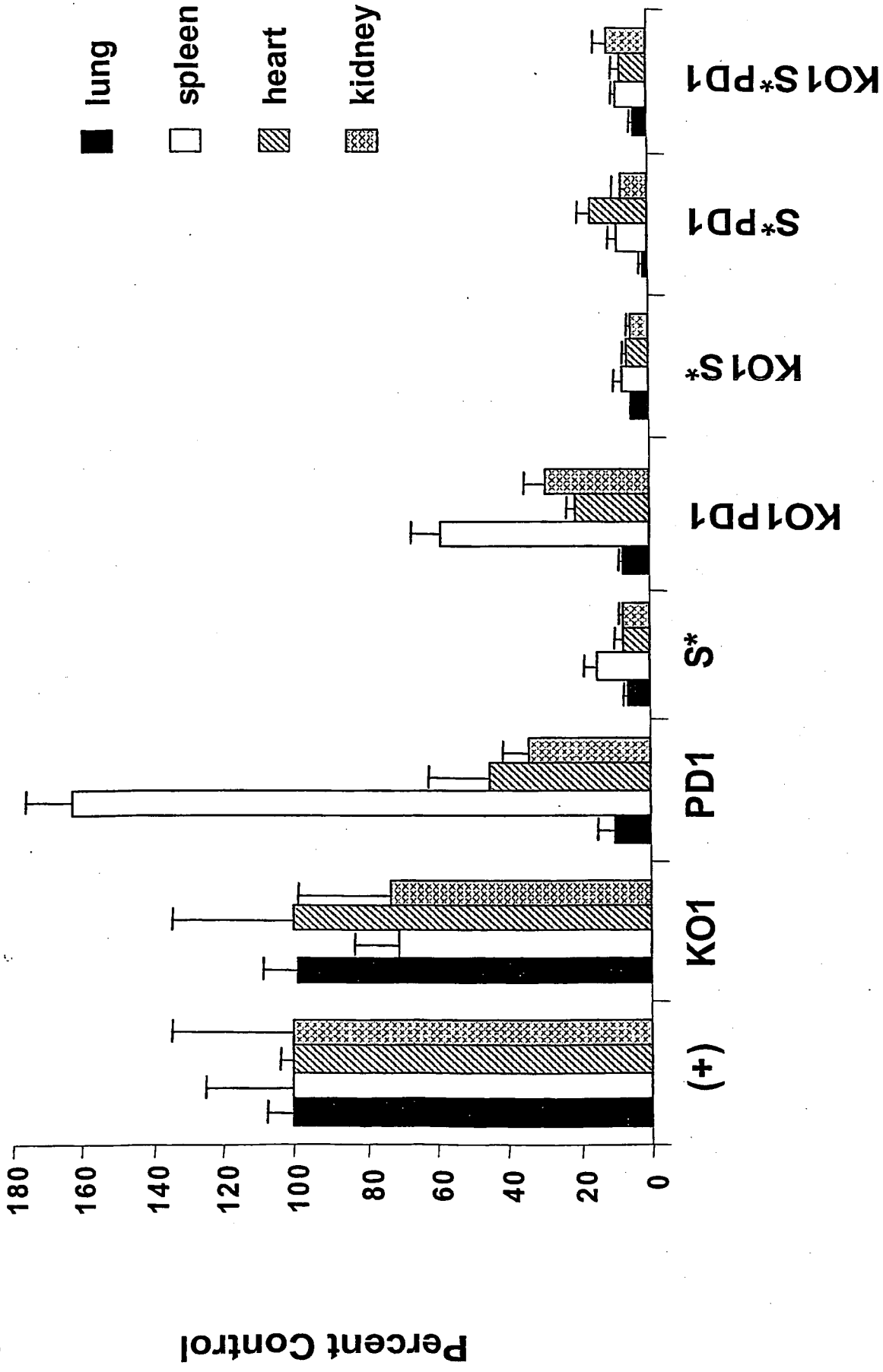

lung
spleen
heart
kidney
Percent Control
180
160
140
120
100
80
60
40
20
0
(+)
KO1
PD1
S*
KO1PD1
KO1S*
S*PD1
KO1S*PD1

Figure 25

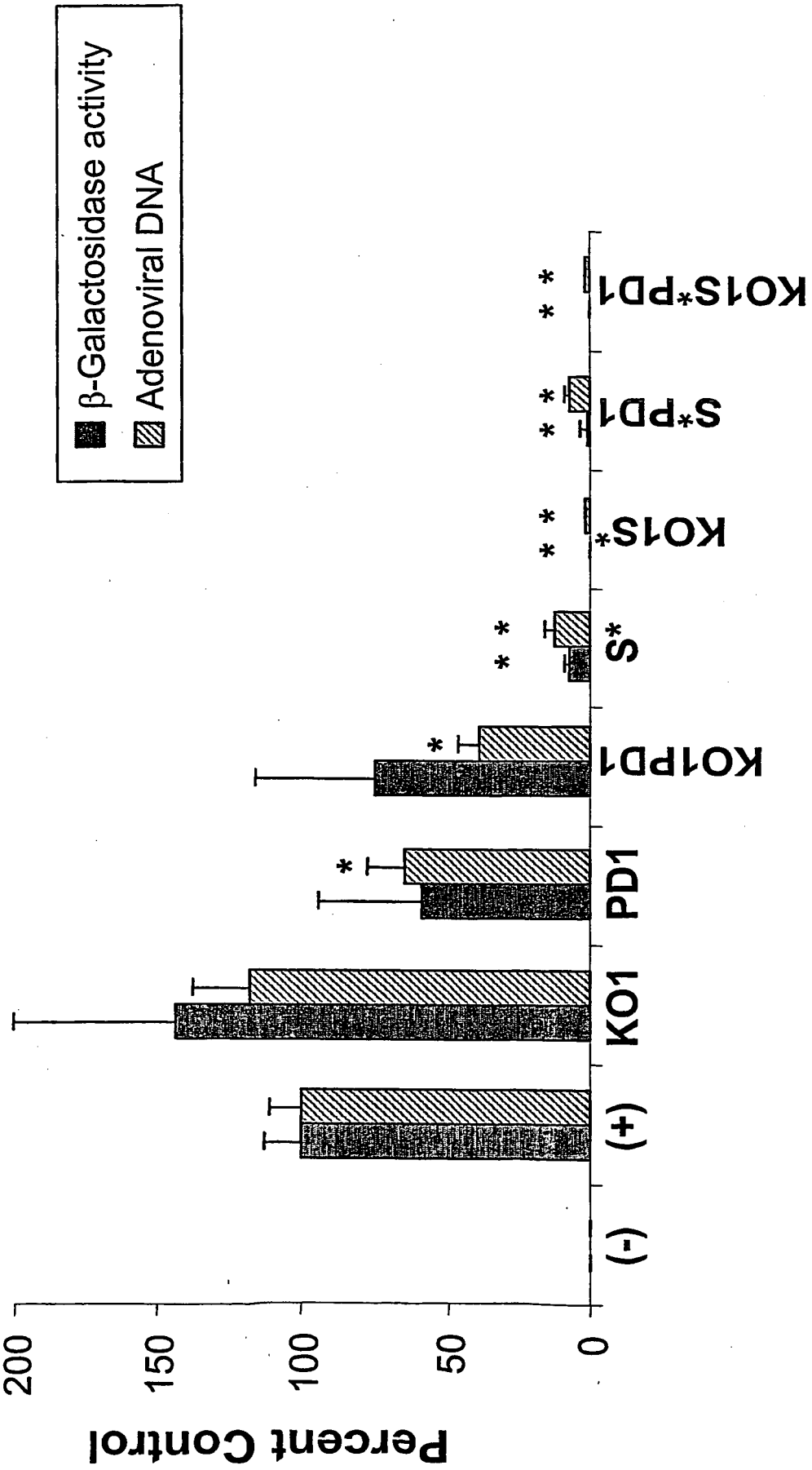

β-Galactosidase activity
Adenoviral DNA
Percent Control
200
150
100
50
0
(-)
(+)
KO1
PD1
KO1PD1
S*
KO1S*
S*PD1
KO1S*PD1

Figure 26

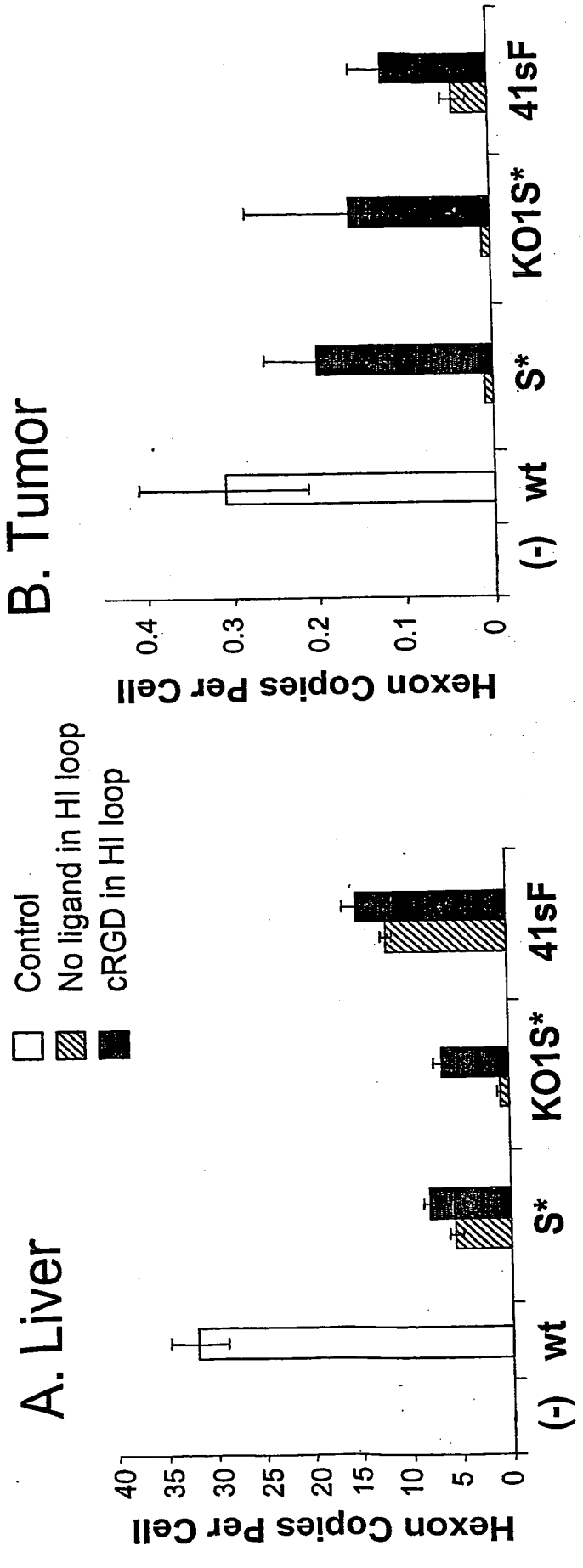

A. Liver
B. Tumor
Control
No ligand in HI loop
cRGD in HI loop
Hexon Copies Per Cell
0
5
10
15
20
25
30
35
40
0
0.1
0.2
0.3
0.4
(-)
wt
S*
KO1S*
41sF

Figure 27

SEQUENCE LISTING

<110> The Scripps Research Institute
Novartis AG
Kaleko, Michael
Nemerow, Glen R.
Smith, Theodore
Stevenson, Susan C.

<120> Fiber Shaft Modifications for Efficient Targeting

<130> 22908-1236PC

<140> Not yet assigned
<141> Herewith

<150> 60/350,388
<151> 2002-01-24

<150> 60/391,967
<151> 2002-06-26

<160> 72

<170> PatentIn version 3.0

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<212> PRT
<213> adenovirus serotype 5

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Lys Lys Thr Lys
1

<210> 2
<211> 1746
<212> DNA
<213> adenovirus serotype 5

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accggtcctc caactgtgcc ttttcttact cctccctttg tatccccaa tgggtttcaa 120
gagagtcccc ctggggtact ctctttgcgc ctatccgaac ctctagttac ctccaatggc 180
atgcttgcgc tcaaaatggg caacggcctc tctctggacg aggccggcaa ccttacctcc 240
caaaatgtaa ccactgtgag cccacctctc aaaaaaacca agtcaaacat aaacctggaa 300
atatctgcac ccctcacagt tacctcagaa gccctaactg tggctgccgc cgcacctcta 360
atggtcgcgg gcaacacact caccatgcaa tcacaggccc cgctaaccgt gcacgactcc 420
aaacttagca ttgccaccca aggacccctc acagtgtcag aaggaaagct agccctgcaa 480
acatcaggcc ccctcaccac caccgatagc agtaccctta ctatcactgc ctcacccct 540
ctaactactg ccactggtag cttgggcatt gacttgaaag agcccattta tacacaaaat 600
ggaaaactag gactaaagta cggggctcct ttgcatgtaa cagacgacct aaacactttg 660
accgtagcaa ctggtccagg tgtgactatt aataatactt ccttgcaaac taaagttact 720
ggagccttgg gttttgattc acaaggcaat atgcaactta atgtagcagg aggactaagg 780
attgattctc aaaacagacg ccttatactt gatgttagtt atccgtttga tgctcaaaac 840
caactaaatc taagactagg acagggccct ctttttataa actcagccca caacttggat 900
attaactaca acaaaggcct ttacttgttt acagcttcaa acaattccaa aaagcttgag 960
gttaacctaa gcactgccaa ggggttgatg tttgacgcta cagccatagc cattaatgca 1020
ggagatgggc ttgaatttgg ttcacctaat gcaccaaaca caaatccccct caaaacaaaa 1080
attggccatg gcctagaatt tgattcaaac aaggctatgg ttcctaaact aggaactggc 1140
cttagttttg acagcacagg tgccattaca gtaggaaaca aaaataatga taagctaact 1200
ttgtggacca caccagctcc agaggctaac tgtagactaa atgcagagaa agatgctaaa 1260

ctgaaatacg agtgggtgga gttcacgctg cccgagggca actactccga gaccatgacc 540
atagacctta tgaacaacgc gatcgtggag cactacttga aagtgggcag acagaacggg 600
gttctggaaa gcgacatcgg ggtaaagttt gacacccgca acttcagact ggggtttgac 660
cccgtcactg gtcttgtcat gcctggggta tatacaaacg aagccttcca tccagacatc 720
attttgctgc caggatgcgg ggtggacttc acccacagcc gcctgagcaa cttgttgggc 780
atccgcaagc ggcaaccctt ccaggagggc tttaggatca cctacgatga tctggagggt 840
ggtaacattc ccgcactgtt ggatgtggac gcctaccagg cgagcttgaa agatgacacc 900
gaacagggcg ggggtggcgc aggcggcagc aacagcagtg gcagcggcgc ggaagagaac 960
tccaacgcgg cagccgcggc aatgcagccg gtggaggaca tgaacgatag ccgcggctac 1020
ccctacgacg tgcccgacta cgcgggcacc agcgccacac gggctgagga gaagcgcgct 1080
gaggccgaag cagcggccga agctgccgcc cccgctgcgc aacccgaggt cgagaagcct 1140
cagaagaaac cggtgatcaa acccctgaca gaggacagca agaaacgcag ttacaaccta 1200
ataagcaatg acagcacctt cacccagtac cgcagctggt accttgcata caactacggc 1260
gaccctcaga ccggaatccg ctcatggacc ctgctttgca ctcctgacgt aacctgcggc 1320
tcggagcagg tctactggtc gttgccagac atgatgcaag accccgtgac cttccgctcc 1380
acgcgccaga tcagcaactt tccggtggtg ggcgccgagc tgttgcccgt gcactccaag 1440
agcttctaca acgaccaggc cgtctactcc caactcatcc gccagtttac ctctctgacc 1500
cacgtgttca atcgctttcc cgagaaccag attttggcgc gcccgccagc ccccaccatc 1560
accaccgtca gtgaaaacgt tcctgctctc acagatcacg ggacgctacc gctgcgcaac 1620
agcatcggag gagtccagcg agtgaccatt actgacgcca gacgccgcac ctgcccctac 1680
gtttacaagg ccctgggcat agtctcgccg cgcgtcctat cgagccgcac tttttga 1737

<210> 5
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<212> DNA
<213> adenovirus serotype 5

<400> 5
gaacaggagg tgagcttaga 20

<210> 6
<211> 43
<212> DNA
<213> adenovirus serotype 5

<400> 6
tccgcctcca tttagtgaac agttaggaga tggagctggt gtg 43

<210> 7
<211> 44
<212> DNA
<213> adenovirus serotype 5

<400> 7
tcactaaatg gaggcggaga tgctaaactc actttggtct taac 44

<210> 8
<211> 20
<212> DNA
<213> adenovirus serotype 5

<400> 8
gtggcaggtt gaatactagg 20

<210> 9
<211> 8
<212> PRT
<213> adenovirus serotype 5

<400> 9
His Ala Ile Arg Gly Asp Thr Phe

<400> 15
ggctccggct ccgagaggtg ggctcacagt ggttacattt 40

<210> 16
<211> 32
<212> DNA
<213> Artificial Sequence
<220>
<223> oligonucleotide for mutation generation

<400> 16
ggagccggag cctcaaacat aaacctggaa at 32
<210> 17
<211> 27
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 17
ctctagaaat ggacggaatt attacag 27

<210> 18
<211> 32
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 18
tcttggtcat ctgcaacaac atgaagatag tg 32

<210> 19
<211> 32
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 19
gttgttgcag atgaccaaga gagtccggct ca 32

<210> 20
<211> 73
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 20
agcaattgaa aaataaacac gttgaaacat aacacaaacg attctttagt tgtcgtcttc 60
tgtaatgtaa gaa 73

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 21

<210> 28
<211> 54
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 28
tgcgcaaaaa caatcaccac gacaatcaca atgtacattg gaagaaatca tacg 54

<210> 29
<211> 54
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 29
acattgtgat tgtcgtggtg attgtttttg cgcatatgcc atacaatttg aatg 54

<210> 30
<211> 10
<212> PRT
<213> Artificial Sequence
<220>
<223> RGD targeting peptide

<400> 30
His Cys Asp Cys Arg Gly Asp Cys Phe Cys
1 5 10

<210> 31
<211> 32
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 31
ttcttttcat ctgcaacaac atgaagatag tg 32

<210> 32
<211> 32
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 32
gttgttgcag atgaaaagaa ccagaattga ag 32

<210> 33
<211> 73
<212> DNA
<213> Artificial Sequence
<220>
<223> amplification primer

<400> 33
tgcaattgaa aaataaacac gttgaaacat aacacaaacg attctttatt cttcagttat 60
gtagcaaaat aca 73

<213> Artificial Sequence
<220>
<223> amplification primer

<400> 40
ttacatgcac atctcgggcc aggac 25

<210> 41
<211> 7607
<212> DNA
<213> Artificial Sequence

<220>
<223> Plasmid GRE5-E1-SV40-Hygro

<400> 41
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ttctagctac tttattagat ccgctgtaca ggatgttcta gctactttat tagatccgct 120
gtacaggatg ttctagctac tttattagat ccgtgtacag gatgttctag ctactttatt 180
agatcgatct cctggccgtt cggggtcaaa aaccaggttt ggctataaaa gggggtgggg 240
gcgcgttcgt cctcactctc ttccgcatcg ctgtctgcga gggccaggat cgatcctgag 300
aacttcaggg tgagtttggg gacccttgat tgttctttct ttttcgctat tgtaaaattc 360
atgttatatg gaggggggcaa agttttcagg gtgttgttta gaatgggaag atgtcccttg 420
tatcaccatg gaccctcatg ataatttttgt ttctttcact ttctactctg ttgacaacca 480
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<213> Artificial Sequence

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<223> 5F KO1

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Ala Pro Leu His Val Thr Asp Asp Leu Asn Thr Leu Thr Val Ala Thr
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Gly Pro Gly Val Thr Ile Asn Asn Thr Ser Leu Gln Thr Lys Val Thr
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Gly Ala Leu Gly Phe Asp Ser Gln Gly Asn Met Gln Leu Asn Val Ala
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Gly Gly Leu Arg Ile Asp Ser Gln Asn Arg Arg Leu Ile Leu Asp Val
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Ser Tyr Pro Phe Asp Ala Gln Asn Gln Leu Asn Leu Arg Leu Gly Gln
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Gly Pro Leu Phe Ile Asn Ser Ala His Asn Leu Asp Ile Asn Tyr Asn
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Lys Gly Leu Tyr Leu Phe Thr Ala Ser Asn Asn Ser Lys Lys Leu Glu
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Val Asn Leu Ser Thr Ala Lys Gly Leu Met Phe Asp Ala Thr Ala Ile
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Ala Ile Asn Ala Gly Asp Gly Leu Glu Phe Gly Ser Pro Asn Ala Pro
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Asn Thr Asn Pro Leu Lys Thr Lys Ile Gly His Gly Leu Glu Phe Asp
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Ser Asn Lys Ala Met Val Pro Lys Leu Gly Thr Gly Leu Ser Phe Asp
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Lys Asp Ala Lys Leu Thr Leu Val Leu Thr Lys Cys Gly Ser Gln Ile
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Phe Met Pro Asn Leu Ser Ala Tyr Pro Lys Ser His Gly Lys Thr Ala
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<213> Artificial Sequence

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Gly Pro Gly Val Thr Ile Asn Asn Thr Ser Leu Gln Thr Lys Val Thr
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Ser Tyr Pro Phe Asp Ala Gln Asn Gln Leu Asn Leu Arg Leu Gly Gln
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Gly Pro Leu Phe Ile Asn Ser Ala His Asn Leu Asp Ile Asn Tyr Asn
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Lys Asp Ala Lys Leu Thr Leu Val Leu Thr Lys Cys Gly Ser Gln Ile
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485 490 495
Phe Met Pro Asn Leu Ser Ala Tyr Pro Lys Ser His Gly Lys Thr Ala
500 505 510
Lys Ser Asn Ile Val Ser Gln Val Tyr Leu Asn Gly Asp Lys Thr Lys
515 520 525
Pro Val Thr Leu Thr Ile Thr Leu Asn Gly Thr Gln Glu Thr Gly Asp
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His Cys Asp Cys Arg Gly Asp Cys Phe Cys Thr Thr Pro Ser Ala Tyr
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Thr Ser Gly Pro Leu Thr Thr Thr Asp Ser Ser Thr Leu Thr Ile Thr
                165                 170                 175
Ala Ser Pro Pro Leu Thr Thr Ala Thr Gly Ser Leu Gly Ile Asp Leu
            180                 185                 190
Lys Glu Pro Ile Tyr Thr Gln Asn Gly Lys Leu Gly Leu Lys Tyr Gly
        195                 200                 205
Ala Pro Leu His Val Thr Asp Asp Leu Asn Thr Leu Thr Val Ala Thr
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Gly Pro Gly Val Thr Ile Asn Asn Thr Ser Leu Gln Thr Lys Val Thr
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Gly Gly Leu Arg Ile Asp Ser Gln Asn Arg Arg Leu Ile Leu Asp Val
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Ser Tyr Pro Phe Asp Ala Gln Asn Gln Leu Asn Leu Arg Leu Gly Gln
        275                 280                 285
Gly Pro Leu Phe Ile Asn Ser Ala His Asn Leu Asp Ile Asn Tyr Asn
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Lys Gly Leu Tyr Leu Phe Thr Ala Ser Asn Asn Ser Lys Lys Leu Glu
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Val Asn Leu Ser Thr Ala Lys Gly Leu Met Phe Asp Ala Thr Ala Ile
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Ala Ile Asn Ala Gly Asp Gly Leu Glu Phe Gly Ser Pro Asn Ala Pro
            340                 345                 350
Asn Thr Asn Pro Leu Lys Thr Lys Ile Gly His Gly Leu Glu Phe Asp
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Ser Asn Lys Ala Met Val Pro Lys Leu Gly Thr Gly Leu Ser Phe Asp
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Ser Thr Gly Ala Ile Thr Val Gly Asn Lys Asn Asn Asp Lys Leu Thr
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Leu Trp Thr Thr Pro Ala Pro Ser Pro Asn Cys Ser Leu Asn Gly Gly
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Gly Asp Ala Lys Leu Thr Leu Val Leu Thr Lys Cys Gly Ser Gln Ile
            420                 425                 430
Leu Ala Thr Val Ser Val Leu Ala Val Lys Gly Ser Leu Ala Pro Ile
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Ser Gly Thr Val Gln Ser Ala His Leu Ile Ile Arg Phe Asp Glu Asn
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Gly Val Leu Leu Asn Asn Ser Phe Leu Asp Pro Glu Tyr Trp Asn Phe
465                 470                 475                 480
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Phe Met Pro Asn Leu Ser Ala Tyr Pro Lys Ser His Gly Lys Thr Ala
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Lys Ser Asn Ile Val Ser Gln Val Tyr Leu Asn Gly Asp Lys Thr Lys
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Pro Val Thr Leu Thr Ile Thr Leu Asn Gly Thr Gln Glu Thr Gly Asp
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Thr Thr Pro Ser Ala Tyr Ser Met Ser Phe Ser Trp Asp Trp Ser Gly
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His Asn Tyr Ile Asn Glu Ile Phe Ala Thr Ser Ser Tyr Thr Phe Ser
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Tyr Ile Ala Gln Glu
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Lys Asp Ala Lys Leu Thr Leu Val Leu Thr Lys Cys Gly Ser Gln Ile
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Lys Ser Asn Ile Val Ser Gln Val Tyr Leu Asn Gly Asp Lys Thr Lys
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His Asn Tyr Ile Asn Glu Ile Phe Ala Thr Ser Ser Tyr Thr Phe Ser
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Tyr Ile Ala Gln Glu

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Gln Asn Val Thr Thr Val Ser Pro Pro Leu Gly Ala Gly Ala Ser Asn
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Ile Asn Leu Glu Ile Ser Ala Pro Leu Thr Val Thr Ser Glu Ala Leu
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Lys Met Gly Asn Gly Leu Ser Leu Asp Glu Ala Gly Asn Leu Thr Ser
65                  70                  75                  80
Gln Asn Val Thr Thr Val Ser Pro Pro Leu Gly Ala Gly Ala Ser Asn
                85                  90                  95
Ile Asn Leu Glu Ile Ser Ala Pro Leu Thr Val Thr Ser Glu Ala Leu
            100                 105                 110
Thr Val Ala Ala Ala Ala Ala Pro Leu Met Val Ala Gly Asn Thr Leu Thr
        115                 120                 125
Met Gln Ser Gln Ala Pro Leu Thr Val His Asp Ser Lys Leu Ser Ile
    130                 135                 140
Ala Thr Gln Gly Pro Leu Thr Val Ser Glu Gly Lys Leu Ala Leu Gln
145                 150                 155                 160
Thr Ser Gly Pro Leu Thr Thr Thr Asp Ser Ser Thr Leu Thr Ile Thr
                165                 170                 175
Ala Ser Pro Pro Leu Thr Thr Ala Thr Gly Ser Leu Gly Ile Asp Leu
            180                 185                 190
Lys Glu Pro Ile Tyr Thr Gln Asn Gly Lys Leu Gly Leu Lys Tyr Gly
        195                 200                 205
Ala Pro Leu His Val Thr Asp Asp Leu Asn Thr Leu Thr Val Ala Thr
    210                 215                 220
Gly Pro Gly Val Thr Ile Asn Asn Thr Ser Leu Gln Thr Lys Val Thr
225                 230                 235                 240
Gly Ala Leu Gly Phe Asp Ser Gln Gly Asn Met Gln Leu Asn Val Ala
                245                 250                 255
Gly Gly Leu Arg Ile Asp Ser Gln Asn Arg Arg Leu Ile Leu Asp Val
            260                 265                 270
Ser Tyr Pro Phe Asp Ala Gln Asn Gln Leu Asn Leu Arg Leu Gly Gln
        275                 280                 285
Gly Pro Leu Phe Ile Asn Ser Ala His Asn Leu Asp Ile Asn Tyr Asn
    290                 295                 300
Lys Gly Leu Tyr Leu Phe Thr Ala Ser Asn Asn Ser Lys Lys Leu Glu
305                 310                 315                 320
Val Asn Leu Ser Thr Ala Lys Gly Leu Met Phe Asp Ala Thr Ala Ile
                325                 330                 335
Ala Ile Asn Ala Gly Asp Gly Leu Glu Phe Gly Ser Pro Asn Ala Pro
            340                 345                 350
Asn Thr Asn Pro Leu Lys Thr Lys Ile Gly His Gly Leu Glu Phe Asp
        355                 360                 365
Ser Asn Lys Ala Met Val Pro Lys Leu Gly Thr Gly Leu Ser Phe Asp
    370                 375                 380
Ser Thr Gly Ala Ile Thr Val Gly Asn Lys Asn Asn Asp Lys Leu Thr
385                 390                 395                 400
Leu Trp Thr Thr Pro Ala Pro Glu Ala Asn Cys Arg Leu Asn Ala Glu
                405                 410                 415
Lys Asp Ala Lys Leu Thr Leu Val Leu Thr Lys Cys Gly Ser Gln Ile
            420                 425                 430
Leu Ala Thr Val Ser Val Leu Ala Val Lys Gly Ser Leu Ala Pro Ile
        435                 440                 445
Ser Gly Thr Val Gln Ser Ala His Leu Ile Ile Arg Phe Asp Glu Asn
    450                 455                 460
Gly Val Leu Leu Asn Asn Ser Phe Leu Asp Pro Glu Tyr Trp Asn Phe
465                 470                 475                 480
Arg Asn Gly Asp Leu Thr Glu Gly Thr Ala Tyr Thr Asn Ala Val Gly
                485                 490                 495
Phe Met Pro Asn Leu Ser Ala Tyr Pro Lys Ser His Gly Lys Thr Ala
            500                 505                 510
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```
                100                 105                 110
Lys Leu Gly Asn Gly Leu Lys Phe Asn Asn Gly Asp Ile Cys Ile Lys
            115                 120                 125
Asp Ser Ile Asn Thr Leu Trp Thr Gly Ile Asn Pro Pro Pro Asn Cys
    130                 135                 140
Gln Ile Val Glu Asn Thr Asn Thr Asn Asp Gly Lys Leu Thr Leu Val
145                 150                 155                 160
Leu Val Lys Asn Gly Gly Leu Val Asn Gly Tyr Val Ser Leu Val Gly
                165                 170                 175
Val Ser Asp Thr Val Asn Gln Met Phe Thr Gln Lys Thr Ala Asn Ile
            180                 185                 190
Gln Leu Arg Leu Tyr Phe Asp Ser Ser Gly Asn Leu Leu Thr Glu Glu
        195                 200                 205
Ser Asp Leu Lys Ile Pro Leu Lys Asn Lys Ser Ser Thr Ala Thr Ser
    210                 215                 220
Glu Thr Val Ala Ser Ser Lys Ala Phe Met Pro Ser Thr Thr Ala Tyr
225                 230                 235                 240
Pro Phe Asn Thr Thr Thr Arg Asp Ser Glu Asn Tyr Ile His Gly Ile
                245                 250                 255
Cys Tyr Tyr Met Thr Ser Tyr Asp Arg Ser Leu Phe Pro Leu Asn Ile
            260                 265                 270
Ser Ile Met Leu Asn Ser Arg Met Ile Ser Ser Asn Val Ala Tyr Ala
        275                 280                 285
Ile Gln Phe Glu Trp Asn Leu Asn Ala Ser Glu Ser Pro Glu Ser Asn
    290                 295                 300
Ile Ala Thr Leu Thr Thr Ser Pro Phe Phe Phe Ser Tyr Ile Thr Glu
305                 310                 315                 320
Asp Asp Glu
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<210> 61
<211> 1002
<212> DNA
<213> Artificial Sequence

<220>
<223> 35F RGD

<400> 61

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atgaccaaga gagtccggct cagtgactcc ttcaaccctg tctacccctа tgaagatgaa 60
agcacctccc aacacccctt tataaaccca gggtttattt ccccaaatgg cttcacacaa 120
agcccagacg gagttcttac tttaaaatgt ttaaccccac taacaaccac aggcggatct 180
ctacagctaa aagtgggagg gggacttaca gtggatgaca ctgatggtac cttacaagaa 240
aacatacgtg ctacagcacc cattactaaa aataatcact ctgtagaact atccattgga 300
aatggattag aaactcaaaa caataaacta tgtgccaaat tgggaaatgg gttaaaattt 360
aacaacggtg acatttgtat aaaggatagt attaacacct tatggactgg aataaaccct 420
ccacctaact gtcaaattgt ggaaaacact aatacaaatg atggcaaact tactttagta 480
ttagtaaaaa atggagggct tgttaatggc tacgtgtctc tagttggtgt atcagacact 540
gtgaaccaaa tgttcacaca aaagacagca aacatccaat taagattata ttttgactct 600
tctggaaatc tattaactga ggaatcagac ttaaaaattc cacttaaaaa taaatcttct 660
acagcgacca gtgaaactgt agccagcagc aaagccttta tgccaagtac tacagcttat 720
cccttcaaca ccactactag ggatagtgaa aactacattc atggaatatg ttactacatg 780
actagttatg atagaagtct atttcccttg aacatttcta taatgctaaa cagccgtatg 840
atttcttcca atgtacattg tgattgtcgt ggtgattgtt tttgcgcata tgccatacaa 900
tttgaatgga atctaaatgc aagtgaatct ccagaaagca acatagctac gctgaccaca 960
tccccctttt tcttttctta cattacagaa gacgacgaat aa                   1002
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<210> 62
<211> 333
<212> PRT

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cccccaggag ttttagcact caagtacact gaccccatta ctaccaatgc taagcatgag 180
cttactttaa aacttggaag caacataact ttagaaaatg ggttactttc ggccacagtt 240
cccactgttt ctcctcccct tacaaacagt aacaactccc tgggtttagc cacatccgct 300
cccatagctg tatcagctaa ctctctcaca ttggccaccg ccgcaccact gacagtaagc 360
aacaaccagc ttagtattaa cgcgggcaga ggtttagtta taactaacaa tgccttaaca 420
gttaatccta ccggagcgct aggtttcaat aacacaggag ctttacaatt aaatgctgca 480
ggaggaatga gagtggacgg tgccaactta attcttcatg tagcatatcc ctttgaagca 540
atcaaccagc taacactgcg attagaaaac gggttagaag taaccagcgg aggaaagctt 600
aacgttaagt tgggatcagg cctccaattt gacagtaacg gacgcattgc tattagtaat 660
agcaaccgaa ctcgaagtgt accatccctc actaccattt ggtctatctc gcctacgcct 720
aactgctcca tttatgaaac ccaagatgca aacctatttc tttgtctaac taaaaacgga 780
gctcacgtat taggtactat aacaatcaaa ggtcttaaag gagcactgcg ggaaatgcac 840
gataacgctc tatctttaaa acttcccttt gacaatcagg gaaatttact taactgtgcc 900
ttggaatcat ccacctggcg ttaccaggaa accaacgcag tggcctctaa tgccttaaca 960
tttatgccca acagtacagt gtatccacga aacaaaaccg ctcacccggg caacatgctc 1020
atccaaatct cgcctaacat caccttcagt gtcgtctaca acgagataaa cagtgggtat 1080
gcttttactt ttaaatggtc agccgaaccg ggaaaacctt ttcacccacc taccgctgta 1140
ttttgctaca taactgaaga ataa                                       1164
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<210> 64
<211> 387
<212> PRT
<213> Artificial Sequence

<220>
<223> 41sF

<400> 64

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Met Lys Arg Thr Arg Ile Glu Asp Asp Phe Asn Pro Val Tyr Pro Tyr
 1               5                  10                  15
Asp Thr Phe Ser Thr Pro Ser Ile Pro Tyr Val Ala Pro Pro Phe Val
            20                  25                  30
Ser Ser Asp Gly Leu Gln Glu Lys Pro Pro Gly Val Leu Ala Leu Lys
        35                  40                  45
Tyr Thr Asp Pro Ile Thr Thr Asn Ala Lys His Glu Leu Thr Leu Lys
    50                  55                  60
Leu Gly Ser Asn Ile Thr Leu Glu Asn Gly Leu Leu Ser Ala Thr Val
65                  70                  75                  80
Pro Thr Val Ser Pro Pro Leu Thr Asn Ser Asn Asn Ser Leu Gly Leu
                85                  90                  95
Ala Thr Ser Ala Pro Ile Ala Val Ser Ala Asn Ser Leu Thr Leu Ala
            100                 105                 110
Thr Ala Ala Pro Leu Thr Val Ser Asn Asn Gln Leu Ser Ile Asn Ala
        115                 120                 125
Gly Arg Gly Leu Val Ile Thr Asn Asn Ala Leu Thr Val Asn Pro Thr
    130                 135                 140
Gly Ala Leu Gly Phe Asn Asn Thr Gly Ala Leu Gln Leu Asn Ala Ala
145                 150                 155                 160
Gly Gly Met Arg Val Asp Gly Ala Asn Leu Ile Leu His Val Ala Tyr
                165                 170                 175
Pro Phe Glu Ala Ile Asn Gln Leu Thr Leu Arg Leu Glu Asn Gly Leu
            180                 185                 190
Glu Val Thr Ser Gly Gly Lys Leu Asn Val Lys Leu Gly Ser Gly Leu
        195                 200                 205
Gln Phe Asp Ser Asn Gly Arg Ile Ala Ile Ser Asn Ser Asn Arg Thr
    210                 215                 220
Arg Ser Val Pro Ser Leu Thr Thr Ile Trp Ser Ile Ser Pro Thr Pro
225                 230                 235                 240
Asn Cys Ser Ile Tyr Glu Thr Gln Asp Ala Asn Leu Phe Leu Cys Leu
                245                 250                 255
Thr Lys Asn Gly Ala His Val Leu Gly Thr Ile Thr Ile Lys Gly Leu
```

```
                20                  25                  30
Ser Ser Asp Gly Leu Gln Glu Lys Pro Pro Gly Val Leu Ala Leu Lys
            35                  40                  45
Tyr Thr Asp Pro Ile Thr Thr Asn Ala Lys His Glu Leu Thr Leu Lys
        50                  55                  60
Leu Gly Ser Asn Ile Thr Leu Glu Asn Gly Leu Leu Ser Ala Thr Val
65                  70                  75                  80
Pro Thr Val Ser Pro Pro Leu Thr Asn Ser Asn Asn Ser Leu Gly Leu
                85                  90                  95
Ala Thr Ser Ala Pro Ile Ala Val Ser Ala Asn Ser Leu Thr Leu Ala
            100                 105                 110
Thr Ala Ala Pro Leu Thr Val Ser Asn Asn Gln Leu Ser Ile Asn Ala
        115                 120                 125
Gly Arg Gly Leu Val Ile Thr Asn Asn Ala Leu Thr Val Asn Pro Thr
    130                 135                 140
Gly Ala Leu Gly Phe Asn Asn Thr Gly Ala Leu Gln Leu Asn Ala Ala
145                 150                 155                 160
Gly Gly Met Arg Val Asp Gly Ala Asn Leu Ile Leu His Val Ala Tyr
                165                 170                 175
Pro Phe Glu Ala Ile Asn Gln Leu Thr Leu Arg Leu Glu Asn Gly Leu
            180                 185                 190
Glu Val Thr Ser Gly Gly Lys Leu Asn Val Lys Leu Gly Ser Gly Leu
        195                 200                 205
Gln Phe Asp Ser Asn Gly Arg Ile Ala Ile Ser Asn Ser Asn Arg Thr
    210                 215                 220
Arg Ser Val Pro Ser Leu Thr Thr Ile Trp Ser Ile Ser Pro Thr Pro
225                 230                 235                 240
Asn Cys Ser Ile Tyr Glu Thr Gln Asp Ala Asn Leu Phe Leu Cys Leu
                245                 250                 255
Thr Lys Asn Gly Ala His Val Leu Gly Thr Ile Thr Ile Lys Gly Leu
            260                 265                 270
Lys Gly Ala Leu Arg Glu Met His Asp Asn Ala Leu Ser Leu Lys Leu
        275                 280                 285
Pro Phe Asp Asn Gln Gly Asn Leu Leu Asn Cys Ala Leu Glu Ser Ser
    290                 295                 300
Thr Trp Arg Tyr Gln Glu Thr Asn Ala Val Ala Ser Asn Ala Leu Thr
305                 310                 315                 320
Phe Met Pro Asn Ser Thr Val Tyr Pro Arg Asn Lys Thr Ala His Pro
                325                 330                 335
Gly Asn Met Leu Ile Gln Ile Ser Pro Asn Ile Thr Phe Ser Val Val
            340                 345                 350
Tyr Asn Glu Ile Asn Cys Asp Cys Arg Gly Asp Cys Phe Cys Thr Ser
        355                 360                 365
Gly Tyr Ala Phe Thr Phe Lys Trp Ser Ala Glu Pro Gly Lys Pro Phe
    370                 375                 380
His Pro Pro Thr Ala Val Phe Cys Tyr Ile Thr Glu Glu
385                 390                 395
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<210> 67
<211> 1737
<212> DNA
<213> Artificial Sequence

<220>
<223> Ad5 PD1 penton

<400> 67
atgcggcgcg cggcgatgta tgaggaaggt cctcctccct cctacgagag tgtggtgagc 60
gcggcgccag tggcggcggc gctgggttct ccccttcgatg ctcccctgga cccgccgttt 120
gtgcctccgc ggtacctgcg gcctaccggg gggagaaaca gcatccgtta ctctgagttg 180

195 200 205
Lys Phe Asp Thr Arg Asn Phe Arg Leu Gly Phe Asp Pro Val Thr Gly
210 215 220
Leu Val Met Pro Gly Val Tyr Thr Asn Glu Ala Phe His Pro Asp Ile
225 230 235 240
Ile Leu Leu Pro Gly Cys Gly Val Asp Phe Thr His Ser Arg Leu Ser
245 250 255
Asn Leu Leu Gly Ile Arg Lys Arg Gln Pro Phe Gln Glu Gly Phe Arg
260 265 270
Ile Thr Tyr Asp Asp Leu Glu Gly Gly Asn Ile Pro Ala Leu Leu Asp
275 280 285
Val Asp Ala Tyr Gln Ala Ser Leu Lys Asp Asp Thr Glu Gln Gly Gly
290 295 300
Gly Gly Ala Gly Gly Ser Asn Ser Ser Gly Ser Gly Ala Glu Glu Asn
305 310 315 320
Ser Asn Ala Ala Ala Ala Ala Met Gln Pro Val Glu Asp Met Asn Asp
325 330 335
Ser Arg Gly Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Gly Thr Ser Ala
340 345 350
Thr Arg Ala Glu Glu Lys Arg Ala Glu Ala Glu Ala Ala Ala Glu Ala
355 360 365
Ala Ala Pro Ala Ala Gln Pro Glu Val Glu Lys Pro Gln Lys Lys Pro
370 375 380
Val Ile Lys Pro Leu Thr Glu Asp Ser Lys Lys Arg Ser Tyr Asn Leu
385 390 395 400
Ile Ser Asn Asp Ser Thr Phe Thr Gln Tyr Arg Ser Trp Tyr Leu Ala
405 410 415
Tyr Asn Tyr Gly Asp Pro Gln Thr Gly Ile Arg Ser Trp Thr Leu Leu
420 425 430
Cys Thr Pro Asp Val Thr Cys Gly Ser Glu Gln Val Tyr Trp Ser Leu
435 440 445
Pro Asp Met Met Gln Asp Pro Val Thr Phe Arg Ser Thr Arg Gln Ile
450 455 460
Ser Asn Phe Pro Val Val Gly Ala Glu Leu Leu Pro Val His Ser Lys
465 470 475 480
Ser Phe Tyr Asn Asp Gln Ala Val Tyr Ser Gln Leu Ile Arg Gln Phe
485 490 495
Thr Ser Leu Thr His Val Phe Asn Arg Phe Pro Glu Asn Gln Ile Leu
500 505 510
Ala Arg Pro Pro Ala Pro Thr Ile Thr Thr Val Ser Glu Asn Val Pro
515 520 525
Ala Leu Thr Asp His Gly Thr Leu Pro Leu Arg Asn Ser Ile Gly Gly
530 535 540
Val Gln Arg Val Thr Ile Thr Asp Ala Arg Arg Arg Thr Cys Pro Tyr
545 550 555 560
Val Tyr Lys Ala Leu Gly Ile Val Ser Pro Arg Val Leu Ser Ser Arg
565 570 575
Thr Phe

<210> 69
<211> 1773
<212> DNA
<213> Artificial Sequence

<220>
<223> 5TS35H

<400> 69
atgaagcgcg caagaccgtc tgaagatacc ttcaaccccg tgtatccata tgacacggaa 60

Ala Ser Pro Pro Leu Thr Thr Ala Thr Gly Ser Leu Gly Ile Asp Leu
180 185 190
Lys Glu Pro Ile Tyr Thr Gln Asn Gly Lys Leu Gly Leu Lys Tyr Gly
195 200 205
Ala Pro Leu His Val Thr Asp Asp Leu Asn Thr Leu Thr Val Ala Thr
210 215 220
Gly Pro Gly Val Thr Ile Asn Asn Thr Ser Leu Gln Thr Lys Val Thr
225 230 235 240
Gly Ala Leu Gly Phe Asp Ser Gln Gly Asn Met Gln Leu Asn Val Ala
245 250 255
Gly Gly Leu Arg Ile Asp Ser Gln Asn Arg Arg Leu Ile Leu Asp Val
260 265 270
Ser Tyr Pro Phe Asp Ala Gln Asn Gln Leu Asn Leu Arg Leu Gly Gln
275 280 285
Gly Pro Leu Phe Ile Asn Ser Ala His Asn Leu Asp Ile Asn Tyr Asn
290 295 300
Lys Gly Leu Tyr Leu Phe Thr Ala Ser Asn Asn Ser Lys Lys Leu Glu
305 310 315 320
Val Asn Leu Ser Thr Ala Lys Gly Leu Met Phe Asp Ala Thr Ala Ile
325 330 335
Ala Ile Asn Ala Gly Asp Gly Leu Glu Phe Gly Ser Pro Asn Ala Pro
340 345 350
Asn Thr Asn Pro Leu Lys Thr Lys Ile Gly His Gly Leu Glu Phe Asp
355 360 365
Ser Asn Lys Ala Met Val Pro Lys Leu Gly Thr Gly Leu Ser Phe Asp
370 375 380
Ser Thr Gly Ala Ile Thr Val Gly Asn Lys Asn Asn Asp Lys Leu Thr
385 390 395 400
Leu Trp Thr Gly Ile Asn Pro Pro Pro Asn Cys Gln Ile Val Glu Asn
405 410 415
Thr Asn Thr Asn Asp Gly Lys Leu Thr Leu Val Leu Val Lys Asn Gly
420 425 430
Gly Leu Val Asn Gly Tyr Val Ser Leu Val Gly Val Ser Asp Thr Val
435 440 445
Asn Gln Met Phe Thr Gln Lys Thr Ala Asn Ile Gln Leu Arg Leu Tyr
450 455 460
Phe Asp Ser Ser Gly Asn Leu Leu Thr Glu Glu Ser Asp Leu Lys Ile
465 470 475 480
Pro Leu Lys Asn Lys Ser Ser Thr Ala Thr Ser Glu Thr Val Ala Ser
485 490 495
Ser Lys Ala Phe Met Pro Ser Thr Thr Ala Tyr Pro Phe Asn Thr Thr
500 505 510
Thr Arg Asp Ser Glu Asn Tyr Ile His Gly Ile Cys Tyr Tyr Met Thr
515 520 525
Ser Tyr Asp Arg Ser Leu Phe Pro Leu Asn Ile Ser Ile Met Leu Asn
530 535 540
Ser Arg Met Ile Ser Ser Asn Val Ala Tyr Ala Ile Gln Phe Glu Trp
545 550 555 560
Asn Leu Asn Ala Ser Glu Ser Pro Glu Ser Asn Ile Ala Thr Leu Thr
565 570 575
Thr Ser Pro Phe Phe Phe Ser Tyr Ile Thr Glu Asp Asp Glu
580 585 590

<210> 71
<211> 945
<212> DNA
<213> Artificial Sequence

<220>
<223> 35TS5H

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Gln Glu Thr Gly Asp Thr Thr Pro Ser Ala Tyr Ser Met Ser Phe Ser
           275                 280                 285
Trp Asp Trp Ser Gly His Asn Tyr Ile Asn Glu Ile Phe Ala Thr Ser
       290                 295                 300
Ser Tyr Thr Phe Ser Tyr Ile Ala Gln Glu
305                 310
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